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Human Antibody Response to Three Meningococcal Outer Membrane Antigens: Comparison by Specific Hemagglutination Assays

W. D. ZOLLINGER, C. L. PENNINGTON, AND M. S. ARTENSTEIN

Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012

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Three cell surface antigens, protein, lipopolysaccharide, and polysaccharide, were purified from group B and group C strains of *Neisseria meningitidis* representing a variety of serotypes. Chemical analysis indicated that cross-contamination was on the order of 1%. Sensitization of sheep erythrocytes with these antigens resulted in highly specific passive hemagglutination assays for the three kinds of antigens. Paired human sera from several groups of individuals were tested by hemagglutination for antibody against each of the antigens. Patients with group B or C systemic meningococcal disease showed increases in antibody titer against all three kinds of antigens, but the antibody response to B polysaccharide was low compared with the response to C polysaccharide. Nasopharyngeal carriers of group B meningococci showed significant increases in titer only against the protein antigens, and noncarriers who received a C-polysaccharide vaccine had a specific response to the C polysaccharide. A given protein or lipopolysaccharide antigen reacted on the average equally well with either group B or C convalescent sera. These results suggest that all three antigens may play a role in the broad human immunity following natural infection.

Both capsular polysaccharides and noncapsular antigens of the meningococcus appear to play a role in human immunity (8, 13). Although the polysaccharides of serogroups A, B, and C induce group-specific antibody responses (2, 9) in humans, natural infection results in cross-reactive antibodies as measured by bactericidal reactions and immunofluorescence (2, 8). The exact nature of these noncapsular cross-reactive antigens is not completely defined as yet, but protein antigens (13) as well as lipopolysaccharides (LPS) (8) have been implicated. In previous studies, the major surface antigens of meningococci have been isolated as a native outer membrane complex (OMC), and polysaccharide, protein, and LPS components were separated (22).

The present investigations were undertaken to determine human antibody responses to these antigens after natural infection. Using purified antigens and passive hemagglutination tests, we found that systemic infection induces antibodies to each of the major antigens.

MATERIALS AND METHODS

Bacterial strains. The strains of *Neisseria meningitidis* were from the culture collection of the Department of Bacterial Diseases, Walter Reed Army Institute of Research. Cultures were preserved in the lyophilized state and used within eight passages of original isolation. The serogroup of each strain is indicated by the letter in parenthesis in the strain designation.

Media and growth conditions were as previously described (22).

Rabbit antisera. Antisera against whole meningococci were made in 4- to 5-lb (about 8.8 to 11.0 kg) New Zealand white rabbits. Three intravenous injections of 0.5 ml were given on days 1, 21, and 42, and the rabbits were bled on day 50. The first injection contained approximately 2×10^7 formalin-killed organisms, and the following two injections contained approximately 5×10^8 live organisms.

Source of human sera. Serum specimens from Army recruits with systemic disease due to group B or C *N. meningitidis* were collected at Fort Dix, N.J., between 1970 and 1972. Acute sera were obtained at the onset of symptoms and before initiation of antibiotic therapy. Convalescent sera were obtained 7 to 30 days after onset of disease. Pre- and postvaccination sera were obtained from recruit volunteers who had received a 50-μg dose of group C vaccine as part of a combined C-vaccine trial and carrier study at Fort Ord, Calif., in 1973. Sera were stored frozen at −30 C and most had been thawed several times before use.

Purification of antigens. OMC was prepared from pelleted organisms by using the following modifications of the previously described methods (22). Organisms suspended in 5 to 10 volumes of TSE buffer [0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.15 N NaCl, 0.01 M ethylenediamine-

tetraacetic acid (EDTA), pH 7.4] were heated at 60 C for 30 min and sheared by mixing in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) at top speed for 3 min at 20 to 25 C. The organisms were pelleted by centrifugation at 16,000 × *g* for 15 min, resuspended in buffer, and sheared in the Omnimixer again. The organisms were pelleted as before, the two supernatants were pooled and twice centrifuged at 16,000 × *g* for 15 min, and the OMC was recovered by centrifugation at 100,000 × *g* for 2 h. The pellets were suspended in distilled water and centrifuged at 10,000 × *g* for 10 min to remove any large debris, and the OMC was repelleted at 100,000 × *g* for 2 h.

The pelleted OMC, which was slightly yellow and transparent, was obtained in amounts ranging from 100 to 200 mg of protein per 50 g of wet, packed cells. The OMC consisted principally of small membrane vesicles and fragments (22). Identification of OMC as outer membrane was based on the following observations. (i) Examination by electron microscopy demonstrated that the cells from which the OMC was extracted remained largely intact but had their outer membranes partially stripped away. (ii) The OMC contained 10 to 25% LPS, which is known to be localized in the outer membrane. (iii) OMC banded as a single peak on isopycnic sucrose density gradients (22). (iv) Comparisons of OMC with outer membrane prepared by isopycnic sucrose density gradient centrifugation of ultrasonically lysed EDTA-lysozyme spheroplasts have demonstrated that they have the same density (1.23 g/cm^3) and the same relatively simple pattern of protein bands on sodium dodecyl sulfate (SDS)-polyacrylamide gels. An inner membrane enriched fraction was found to have a lower density (1.18 g/cm^3), a markedly different and more complex pattern of protein bands on SDS-gels, and 10-fold higher specific activity of succinate dehydrogenase (EC 1.3.99.1) and D-lactate dehydrogenase (EC 1.1.1.28) than outer membrane (W. D. Zollinger, manuscript in preparation).

Protein and LPS were isolated from the preparation of OMC in the following manner. The OMC was precipitated by addition of NaCl to 0.1 M followed by 4 volumes of cold absolute ethanol. The supernatant containing much of the lipid was discarded, and the pelleted precipitate was suspended in water to a concentration of 5 to 10 mg of protein per ml. One-tenth volume of a buffer concentrate containing 0.5 M glycine, 0.01 M EDTA, and 10% sodium deoxycholate (DOC), pH 10.0, was added to the suspension. The pH of the solution was then brought to 11.5 by dropwise addition of 2 N NaOH. The clear solution was immediately applied to a column of Sephadex G-100 equilibrated with GED buffer (0.05 M glycine, 0.001 M EDTA, 0.5% DOC, pH 10.0). The sample was eluted with GED buffer at room temperature, and the fractions were assayed for protein, sialic acid, and 2-keto-3-deoxyoctonic acid (KDO). Those fractions comprising the protein peak and the LPS peak were pooled. The antigens were recovered from the chromatography buffer by one of two methods. (i) The samples were extensively dialyzed against distilled water adjusted to pH 9.0 with a drop of two of ammonium hydroxide and then concentrated to 0.1 volume by ultrafiltration on a PM10 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). Residual bound DOC was removed by adding NaCl to 0.2 N, precipitating the samples with 4 volumes of cold ethanol, and washing the precipitate once with absolute ethanol. (ii) Alternatively, the pH of the pooled fractions was lowered to 5.0 with HCl, and NaCl was added to 0.2 N. The heavy precipitate consisting principally of DOC but including the antigens was removed by centrifugation. The precipitate was washed three times with 10 or more volumes of cold, absolute ethanol, which selectively dissolved the DOC. The antigen remaining as the ethanol-insoluble residue was taken up in a small volume of water. (Procedure [ii] was inefficient for recovery of the LPS fractions.)

The LPS recovered from the column contained a small amount of contaminating protein, which was removed by extracting it with an equal volume of 90% phenol at 68 to 70 C as described by Westphal et al. (21). The LPS was recovered from the aqueous phase by dialysis and lyophilization.

Removal of the 3 to 10% sialic acid (presumably present in the form of capsular polysaccharide) from the protein fraction was accomplished by dissolving the protein in water with the aid of a small amount of 0.1 N NaOH and precipitating the protein at room temperature with 50% trichloroacetic acid added slowly to a final concentration of 25%. The mixture was incubated at room temperature for 1 h and centrifuged at 1,000 × *g*, and the precipitate was washed twice with cold distilled water and dissolved in dilute NaOH at pH 11.5 before dialysis. To summarize, isolation of OMC and purification of the protein and LPS components is as follows. For both LPS and protein pools, place cells in TSE buffer; heat at 65 C for 30 min; shear 3 min in Omnimixer; pellet cells and debris at 3 times 16,000 × *g* for 15 min; pellet OMC twice at 100,000 × *g* for 2 h; suspend pellets in water-OMC; precipitate OMC with ethanol and dissolve in 1% DOC, pH 11.5; chromatograph on Sephadex G-100 in GED buffer, pH 10. Then, for protein pool, remove DOC (method [i] or [ii]); treat with 25% trichloroacetic acid to remove polysaccharide; dialyze and lyophilize. For LPS pool, remove DOC (method [i]; extract with phenol-water to remove protein; dialyze and lyophilize.

Group B and C capsular polysaccharides were purified from the culture supernatant as described by Gotschlich et al. (10) except that in the place of ultracentrifugation and multiple extractions with chloroform-butanol, the partially purified product was dissolved in GED buffer and chromatographed on Sephadex G-200 in GED buffer as described above for OMC. The polysaccharide, which eluted at the void volume, was recovered from the GED buffer as described above (method [i]) and subjected to cold phenol-water extraction to remove any remaining protein. This extraction was carried out as described by Westphal et al. (21) except that the temperature was kept at 8 to 10 C rather than 68 to 70 C.

All antigen preparations were dialyzed against distilled water, lyophilized, and stored under vacuum over P_2O_5 or activated silica gel.

Polyacrylamide gel electrophoresis. Analysis of the protein antigens by polyacrylamide gel electrophoresis in the presence of SDS was carried out as described previously (22).

Chemical assays. Protein was determined by the microbiuret method of Goa (6) and by the method of Lowry et al. (14), using crystallized bovine serum albumin and lysozyme as standards. Hexose was determined by the anthrone reaction as described by Roe (17). The method of Weissbach and Hurwitz (20) as modified by Osborne (16) was used for determination of KDO. Calculations were made by using the extinction coefficient given by Osborne (16). Corrections for interference by sialic acid were made based on the sialic acid content of the sample as determined by the resorcinol method of Svennerholm (18) and the relative reactivity of purified group B and C polysaccharides in the KDO and resorcinol assays.

Indirect hemagglutination assays. Hemagglutination (HA) assays were performed as described by Artenstein et al. (2), using sheep erythrocytes (SRBC) and phosphate-buffered saline (PBS) (0.02 M sodium phosphate, 0.15 M NaCl, pH 7.2) as test buffer in all cases, but the procedure required to achieve optimal sensitization was different for each of the antigens. Sensitization with group polysaccharide was done by using a concentration of 25 μg/ml for both group B and group C polysaccharides. Sensitization with LPS required pretreatment of the LPS with 0.1 N NaOH at 37 C for 2 h. After neutralization with 0.5 N HCl, the LPS was diluted in PBS to 50 μg/ml and sensitization was carried out as for group polysaccharide. Sensitization with the protein antigens required that the SRBC be "tanned." This was done as described by Arbesman et al. (1) except that incubation with tannic acid was at 25 C for 10 min. Due to the insoluble nature of the purified protein antigens, special handling was required to keep them in solution for sensitization. Protein was dissolved at 1 mg/ml in dilute NaOH at pH 11 to 11.5, and the pH was then slowly lowered with HCl until the solution became slightly turbid (usually pH 9.5 to 10). Sodium azide (0.02%) was added, and the antigen was stored at 4 C until used. Just before use, the antigen was diluted with PBS to the optimal concentration for sensitization (about 45 μg/ml) and incubated with an equal volume of 2% tanned SRBC for 10 min at 25 C. The sensitized cells were washed three times and suspended to 0.6% in PBS containing 2% bovine serum albumin. Due to variability among different batches of SRBC in their tendency to autoagglutinate when tanned and coated with the protein antigen, each batch had to be pretested by using a standard antigen and antiserum. Tanned, sensitized SRBC that settled out in a smooth button in the absence of specific antibody were selected for use.

For each preparation of antigen, the optimal sensitizing concentration was determined by grid titration against a standard antiserum. All sera were inactivated at 56 C for 30 min, and human sera were preabsorbed with an equal volume of unsensitized 4% SRBC for 1 h at room temperature to eliminate nonspecific agglutination. Positive and negative controls were included in each test, and the tests were scored after 2 to 4 h at room temperature.

HA inhibition. HA inhibition tests utilized rabbit antiserum diluted to contain 8 units (1 unit of antibody was considered the highest dilution of serum producing maximal agglutination) of antiprotein antibody, 4 units of anti-LPS, or 4 units of anti-group polysaccharide antibody. Antisera were inhibited with serial twofold dilutions of antigens beginning at 1,000 μg/ml. Antigen and antibody were incubated at 37 C for 30 min, after which SRBC sensitized as described above were added and the hemagglutination patterns were read after 2 h at room temperature.

Fractionation of serum on Sephadex G-200. Serum samples (1.5 ml) were chromatographed at 4 C on a column (2.5 by 80 cm) of G-200 equilibrated with buffer containing 0.15 M NaCl and 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4. Fractions (3 ml) were assayed for protein by ultraviolet absorption at 280 nm and for immunoglobulins (Ig) of classes M, G, and A by immunodiffusion using heavy-chain-specific goat anti-human IgM, IgG, or IgA obtained from Hyland Division Travenol Laboratories, Inc., Costa Mesa, Calif.

Preparation of Sepharose-antigen conjugates. Sepharose-antigen conjugates were used as solid immunoadsorbants to study the specificity of the HA reactions. Protein was conjugated directly to cyanogen bromide-activated Sepharose 4B by the method of Cuatrecasas (4). The coupling was carried out with 5 mg of protein per ml at pH 10 and 4 C for 16 h. Sepharose was activated with 100 mg of CNBr per ml of packed Sepharose.

LPS was conjugated to Sepharose by use of the reactive dye APSE hydrogen sulfate as described by Himmelspach and Wrede (12). The etherization was carried out for 1 h at pH 12.5 and 22 C (Na_2CO_3-NaOH) with 1 to 2 mg of LPS and 4 mg of APSE hydrogen sulfate per ml. After separation on a column of Sephadex G-25, the LPS-APSE ether was dissolved in 0.1 N sodium bicarbonate buffer, pH 8.5, and coupled to CNBr-activated Sepharose 4B.

Group B and C polysaccharides were conjugated to aminoethyl Sepharose with the water-soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide by the method of Cuatrecasas (4). Polysaccharide at 4 mg/ml, pH 6.0, was reacted at room temperature for 15 h with an equal volume of packed aminoethyl Sepharose. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to a total concentration of 5 mg/ml.

Treatment of antigen-Sepharose conjugates with Pronase (B grade, Calbiochem, San Diego, Calif.) and Subtilisin (Nutritional Biochemicals, Inc., Cleveland, Ohio) was carried out with 100 μg of enzyme and 0.5 g of packed Sepharose in 1.0 ml of buffer. The buffer was 0.05 M sodium phosphate at pH 7.4 for Pronase treatment and at pH 8.0 for Subtilisin treatment. All Sepharose samples including the control containing no enzyme were incubated at 37 C overnight. The Sepharose samples were then washed on a small filter funnel with 20 volumes each of 0.05 M sodium phosphate, pH 7.4, 0.01 M NaOH, and 0.05 M sodium phosphate, pH 7.4.

Absorption of sera. Absorption of sera with antigens conjugated to Sepharose was carried out by

adding 0.5 ml of packed, conjugated Sepharose to 1 ml of a 1:10 serum dilution and incubating with end-over-end mixing in a stoppered tube for 1 h at room temperature and 2 h at 4 C. Sepharose was removed by filtration on a glass fiber prefilter (Millipore Corp., Bedford, Mass.). In some cases the procedure was repeated.

RESULTS

Purification and characterization of antigens. Gel filtration of solubilized OMC on a column of Sephadex G-100 equilibrated with GED buffer resulted in efficient separation of the protein and LPS (Fig. 1). The protein eluted in one or two peaks, depending on the strain from which the OMC was isolated. The first peak, which eluted at the void volume, was the major peak and was consistently present, whereas the second peak and its resolution from the first were strain dependent. The LPS eluted as a single peak well separated from the bulk of the protein. The small amount of residual protein in the LPS fraction was efficiently removed by extraction with phenol-water. Any group polysaccharide or nucleic acid present in the OMC preparation eluted from the column along with the protein. Normally, nucleic acid was present at a level of only 1 to 2%, but group polysaccharide was usually present in amounts ranging from 3 to 10%. Removal of the group polysaccharide from the protein by treatment with 25% trichloroacetic acid was effective and, though rather harsh, apparently did not destroy the major antigenic determinants of the protein. Attempts to remove residual group polysaccharide from the protein fraction by milder methods were unsuccessful.

The extent of cross-contamination of the purified antigens as determined by chemical analysis is indicated in Table 1. In general, the contamination of any one antigen by either of the others was 1 to 2% or less. The actual contamination of LPS by group-specific polysaccharide is unclear since part of the 3.3% sialic acid found to be present may be an integral part of the LPS structure (11). Contamination of the purified antigens with nucleic acid was found to be 1% or less.

The protein fraction was prepared from OMC isolated from seven test strains chosen on the basis of bactericidal serotyping (7, 13) to repre-

TABLE 1. *Composition of purified antigens*

Assay	Antigen		
	Protein	LPS	Group polysaccharide
Protein	893 ± 30[a]	9.4 ± 4.8	5,8
KDO	1.08 ± 0.37	70 ± 12.3	ND[b]
Hexose	6.1 ± 2.8	259 ± 33.8	6,12
Sialic acid	5.3 ± 2.0	33 ± 10.3	900,960
Nucleic acid ..	10 ± 7.9	<10	<10

[a] Expressed as micrograms per milligram (dry weight). Mean ± standard deviation of five samples from different strains. One sample each of group B and group C polysaccharide was tested.

[b] Not determined.

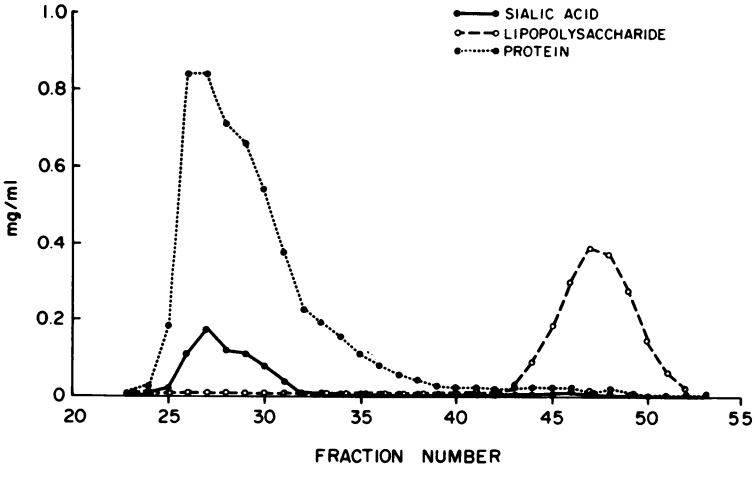

FIG. 1. *Chromatography of OMC from strain 60E(C) on Sephadex G-100. The OMC was precipitated with ethanol and dissolved in glycine buffer containing 1% DOC, pH 11.5, and immediately chromatographed in GED buffer at pH 10. Fractions (18 ml) were assayed for protein, sialic acid, and LPS (based on KDO assuming 7% KDO content).*

sent a variety of serotypes. These antigens, against which human sera were to be tested in passive HA assays (see below), were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2) to compare the number and size distribution of polypeptide chains present in each preparation. The different band patterns obtained with the seven protein fractions support the conclusion from bactericidal serotyping that although some antigenic determinants are shared, none of the strains are identical.

The antigenic relatedness of LPS from the same seven test strains, plus strain 99M(B), was investigated by comparing their ability to react in an HA assay with three rabbit antisera raised against whole meningococci. Only two of the LPS antigens, 138I(C) and 99M(B), gave identical agglutination patterns with the three test sera (Table 2). The LPS from each of the strains, 35E(C), 126E(C), and 99M(B), appears to have at least one determinant not shared by the other two. Each antiserum reacted to some degree with almost every LPS, 99M(B) serum having the broadest spectrum of activity. These results indicate that the LPS antigens are not all identical and that the antigenic determinants of the LPS antigens are independent of serogroup and shared across serogroup lines.

Specificity of indirect HA tests. To investigate the specificity of the polysaccharide, protein, and LPS HA tests, each of the tests was inhibited with serial dilutions of the homologous and heterologous antigens. The tests were highly specific (Table 3). No cross-inhibition of the group C polysaccharide and the LPS such as that described by Mergenhagen et al. (15) was observed. Inhibition by the heterologous antigen required 500 to 1,000 times more antigen than inhibition by the homologous antigen.

These results were confirmed by absorption studies using antigens conjugated to Sepharose

TABLE 2. *Comparison of eight meningococcal LPS antigens by indirect hemagglutination*

Source of LPS used to sensitize erythrocytes	Rabbit antiserum vs. indicated strain[a]		
	35E(C)	126E(C)	99M(B)
35E(C)	64	2	8
126E(C)	<2	64	8
99M(B)	8	2	64
138I(C)	8	2	64
6586(B)	8	2	16
6249(B)	32	2	64
6275(B)	64	8	64
60E(C)	16	8	64

[a] Expressed as reciprocal of highest dilution producing agglutination.

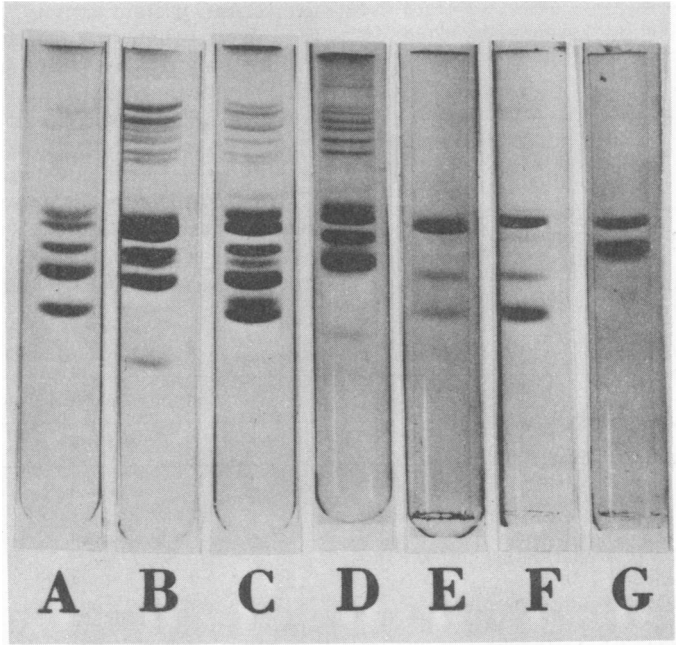

FIG. 2. *Analysis by SDS-polyacrylamide gel electrophoresis of purified outer membrane proteins from seven test strains. The strains from which the proteins were derived were: (A) 6249(B); (B) 6586(B); (C) 6275(B); (D) 138I(C); (E) 60E(C); (F) 35E(C); (G) 126E(C).*

4B (Table 4). Absorption of sera with specific antigen-Sepharose conjugates resulted in selective removal of the homologous antibody, although some loss of the heterologous antigen resulted. Treatment of the protein-Sepharose with Pronase (or Subtilisin) completely destroyed its ability to absorb out anti-protein antibody. Pronase (or Subtilisin) had no effect on the B polysaccharide-Sepharose or the LPS-Sepharose. This demonstrated that the antigenic determinants in the protein fraction were protein or linked to protein, whereas the antigenic activities of the LPS and B-polysaccharide preparations were not due to protein contamination.

Indirect HA activity of major immunoglobulin classes. Several convalescent sera (7 to 34 day) from individuals with systemic meningococcal disease were fractionated on a column of Sephadex G-200 to determine the distribution of HA antibody among the major immunoglobulin classes. An example of results obtained with one 34-day convalescent serum from a patient with group C disease is given in Fig. 3. In this serum, the bulk of the antibody responsible for the hemagglutination observed in the three tests appeared to be of an IgM class. The fractions containing IgG and IgA showed low-titered anti-protein activity. Subsequent assays (not shown) that were performed after concentrating the IgG pooled fractions 15-fold revealed anti-polysaccharide HA activity at a titer of 1:8 and anti-LPS HA activity at a titer of 1:2. The other sera also showed the bulk of the HA activity to be in the IgM-containing fractions.

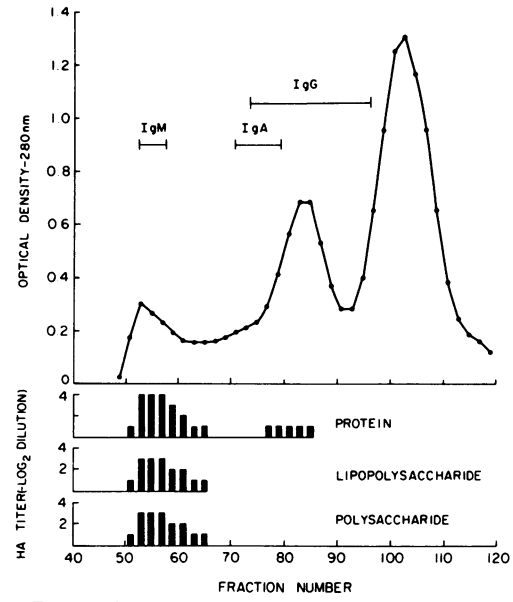

FIG. 3. *Chromatography of convalescent serum from a patient with group C systemic disease on Sephadex G-200. Fractions (3 ml) were assayed for ultraviolet absorbance at 280 nm, for precipitin reactions with antisera specific for human IgA, IgG, and IgM, and for the ability to agglutinate SRBC sensitized with either protein, LPS, or C polysaccharide.*

TABLE 3. *Specificity of hemagglutination assays as evidenced by hemagglutination inhibition*

Inhibiting antigen	Antigen used to sensitize erythrocytes		
	C polysaccharide	138I protein	138I LPS
C polysaccharide	<0.9[a]	>1,000	1,000
138I protein	>1,000	1.8	1,000
138I LPS	>1,000	1,000	1.8

[a] Expressed as minimal concentration of antigen (in micrograms per milliliter) required to inhibit 4 to 8 units of hemagglutination.

Human antibody response to three purified meningococcal antigens. Acute and 7-day convalescent sera from seven individuals with group C systemic disease and seven individuals

TABLE 4. *Specific absorption of hemagglutinating antibodies by using Sepharose-conjugated antigens*

Rabbit antiserum	Erythrocyte sensitizing antigen		
	B polysaccharide	99M protein	99M LPS
Anti-99M(B)	256[a]	1,024	128
Absorbed with:			
B polysaccharide-Sepharose	≤8	1,024	128
Pronase-treated B polysaccharide-Sepharose	≤16	ND[b]	ND
99M protein-Sepharose	128	≤8	64
Pronase-treated 99M protein-Sepharose	ND	1,024	ND
99M LPS-Sepharose	128	512	≤8
Pronase-treated 99M LPS-Sepharose	ND	ND	≤16

[a] Reciprocal of final serum dilution causing hemagglutination.
[b] ND, Not done.

with group B systemic disease were tested in the three HA tests. The sera were tested against group B and C polysaccharides as well as protein and LPS antigens isolated from seven pathogenic test strains. Approximately half of the test strains were group C and half were group B, including isolates from three of the group B patients whose sera were tested. Table 5 presents the mean HA antibody rises for each patient category and each antigen. The anti-polysaccharide responses of the patients with systemic illness were group specific, and the anti-B polysaccharide titers were much lower than anti-C polysaccharide titers. All 14 patients with systemic illness showed fourfold (two tube) or greater antibody increases to most of the protein and LPS antigens. Analysis of variance indicated that there was no significant difference in the responses of the individual patients. In general, there was no serogroup specificity of the responses to the protein and LPS antigens in terms of the serogroup from which the antigen was derived. Convalescent sera from three group B patients had titers to homologous antigens (6586, 6249, 6275) that were no higher than to heterologous antigens.

Human HA antibody response after nasopharyngeal carriage of group B organisms was investigated by testing pre- and post-vaccination sera from two groups of individuals who received a group C polysaccharide vaccine: (i) individuals who were noncarriers at the time of vaccination but became carriers of a group B organism for at least 2 weeks before bleeding at 6 weeks postvaccination; and (ii) individuals who were initially carrier negative and were still carrier negative when bled at 2 weeks postvaccination. Pre- and postvaccination sera from these two groups were tested against group B and C polysaccharides and against protein and LPS antigens isolated from three group B test strains. The mean HA antibody response for the two categories of vaccinees and each antigen is given in Table 6. Group B nasopharyngeal carriers appeared to respond with higher HA antibody titers against protein antigens than against LPS antigens and had virtually no rise against the B polysaccharide. These responses cannot be directly compared with those of the patients with systemic illness because of the difference in the time periods at which sera were obtained. Noncarriers showed no antibody rises to either protein or LPS antigens. The difference in response to group C polysaccharide vaccine between B carriers and noncarriers is accounted for by differences in the time of serum collection.

Figure 4 shows examples of patterns of HA activity of acute and convalescent sera from one patient (C-0537) with group C meningococcal

TABLE 5. *Hemagglutination antibody responses of patients with systemic meningococcal disease to three classes of meningococcal antigens*

Causative organism	No. tested	Antigen class	Strain from which antigens were derived								Mean
			126E(C)	35E(C)	60E(C)	138I(C)	6586(B)	6249(B)	99M(B)	6275(B)	
Group C	7	SSS[a]	-[b]	-	6.3[c] (0–9)	-	-	-	0.25 (0–1)	-	
		Protein	5.7 (0–9)	5.0 (1–8)	3.0 (2–4)	4.0 (3–6)	2.1 (0–4)	3.0 (1–4)	-	4.1 (2–6)	3.9
		LPS	3.3 (1–5)	1.0 (0–3)	-	2.6 (1–5)	2.9 (0–6)	2.6 (1–5)	3.1 (2–6)	2.3 (0–5)	2.5
Group B	7	SSS	-	-	0.1 (0–1)	-	-	-	1.6 (0–4)	-	
		Protein	2.5 (1–7)	5.6 (2–9)	4.0 (2–5)	4.7 (2–7)	2.9 (0–4)	4.0 (3–5)	-	4.2 (1–7)	4.0
		LPS	3.0 (1–4)	1.2 (0–3)	-	3.8 (0–5)	4.3 (0–6)	4.0 (0–5)	3.8 (1–6)	4.4 (1–6)	3.5

[a] SSS, Group polysaccharide.

[b] -, Not determined.

[c] Expressed as geometric mean increase in HA antibody titer: $\log_2$ (reciprocal dilution of convalescent serum) – $\log_2$ (reciprocal dilution of acute serum). The numbers in parentheses indicate the range of the individual titers.

TABLE 6. *Hemagglutination antibody responses of B carriers to three classes of meningococcal antigens*

Category of vaccinees[a]	No. tested	Antigen class	Strain from which antigens were derived				Mean
			60E(C)	6586(B)	6249(B)	99M(B)	
B carriers	10	SSS[b]	4.1[c] (2-7)	-[d]	-	0.4 (0-1)	
		Protein	-	3.5 (0-8)	3.4 (0-7)	3.5 (0-9)	3.5
		LPS	-	1.1 (0-3)	1.4 (0-4)	1.0 (0-4)	1.2
Noncarriers	11	SSS	6.0 (4-7)	-	-	0	
		Protein	-	0	0.1 (0-1)	0.2 (0-1)	0.1
		LPS	-	0.5 (0-1)	0	0.2 (0-1)	0.2

[a] All individuals received group C polysaccharide vaccine on day 1.
[b] SSS, Group polysaccharide.
[c] Geometric mean increases in HA antibody titer: $\log_2$ (reciprocal titer of post-vaccination serum)-$\log_2$ (reciprocal titer of prevaccination serum). The values in parentheses indicate the range of the individual titers.
[d] -, Not tested.

disease and one patient (B-2481) with group B disease. These data were chosen as being representative of the data summarized in Table 5. Although considerable variation in HA titer was observed with the different antigens, a significant rise (fourfold or greater) in antibody titer was observed with nearly all of the antigens.

DISCUSSION

Separation and purification of the major antigens of the outer membrane of *N. meningitidis* in an antigenically active state has made it possible to identify and compare the specific antibody responses of infected humans. The results presented here indicate that individuals who acquire systemic meningococcal infection usually respond by producing antibodies directed against all three antigen classes. Patients with group B disease had only a low-level antibody response to the group B polysaccharide, a finding previously reported from this laboratory by using other assays (2, 19). Antibody titers as previously measured by immunofluorescence, however, were quite comparable to those of patients with group C or Y disease (2). Since immunofluorescence tests used fixed whole organisms as antigen, it was apparent that the patients were developing antibodies to antigens other than the capsular polysaccharides. The current studies provide evidence that these patients develop HA antibodies to outer membrane protein and LPS antigens. Antibodies induced by group B meningococcal infection were, on the average, of equivalent titer to those observed in patients with group C infection, and the degree of reactivity appeared unrelated to the serogroup of meningococcus from which the antigen was extracted.

Group-specific anti-polysaccharide HA responses observed in this study were similar to those noted previously (2). However, a high degree of cross-reactivity was observed among the different protein antigens and, also, among the LPS antigens. Even those antigens considered homologous to the patient (the infecting strain was the source of the antigen) did not react significantly greater with his sera. This extensive cross-reactivity was unexpected, since many of the donor strains were selected to represent different subtypes, a scheme based upon bactericidal tests (7, 13). Indeed, among the donor strains, different protein banding patterns were detected by polyacrylamide gel electrophoresis (Fig. 2).

The degree of antigenic specificity shown by bactericidal assays (5, 7, 13) was not found in HA tests in this study. The lesser specificity observed in the HA tests may be due to different classes of immunoglobulin causing the reactions (HA, IgM; bactericidal, IgG) or to both specific and broadly cross-reactive determinants being

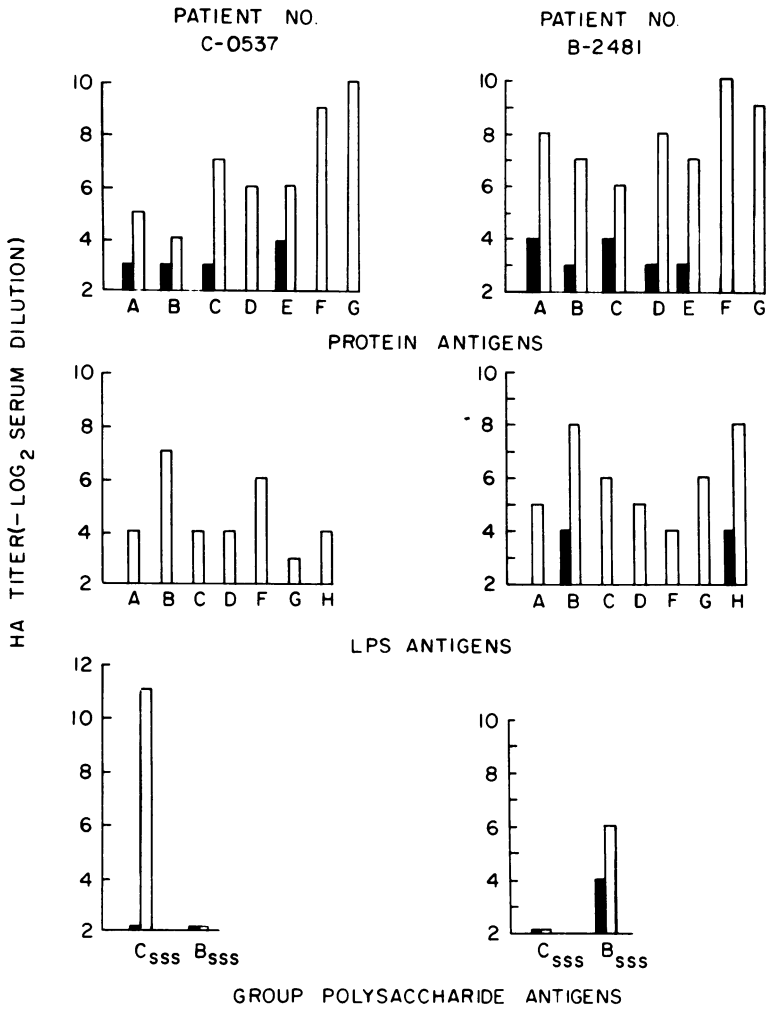

FIG. 4. *HA antibody responses of a patient (C-0537) with group C and a patient (B-2481) with group B systemic meningococcal disease as determined by using a series of meningococcal protein, LPS, and polysaccharide test antigens. Solid bars are acute serum and open bars are convalescent serum. The absence of a solid bar indicates a titer ≤1:4. The protein and LPS antigens were derived from the following strains (serogroup): (A) 6249(B); (B) 6586(B); (C) 6275(B); (D) 138I(B); (E) 60E(C); (F) 35E(C); (G) 126E(C); (H) 99M(B). C_{sss} and B_{sss} indicate group C and group B polysaccharide, respectively.*

present on the protein and LPS antigens. In support of the latter hypothesis, we have found that a much higher degree of specificity is obtained in the protein and LPS HA tests when cross-absorbed rabbit sera are tested (unpublished observations).

Although patients with group B meningococcal disease had approximately equal rises in HA antibody against the protein and LPS antigens, carriers of group B meningococci appeared to respond preferentially to the protein antigens. The reason for this difference is not clear.

It has been suggested that the immune response in man to natural meningococcal infection may be directed in part against antigens other than capsular polysaccharides (8). Protein antigens have been previously implicated (13); the current data suggest a possible role of LPS antigens as well. Studies of the opsonic and bactericidal reactions of antibodies to these antigens will be necessary in order to ascertain their role in immunity and their potential as vaccines.

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