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Recognition of Serogroup A *Neisseria meningitidis* Serotype Antigens by Human Antisera

RENEE J. SUGASAWARA†

Naval Biosciences Laboratory, University of California at Berkeley School of Public Health, Naval Supply Center, Oakland, California 94625

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The antigens of *Neisseria meningitidis* serogroup A which were recognized by human antisera were identified by Western blot and enzyme-linked immunosorbent assay techniques. The components of six prototype strains used for serotyping serogroup A meningococci were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose for immunoperoxidase staining with sera collected from 10 acute-phase and 14 convalescent-phase patients. Six acute-phase sera detected six major antigens having apparent molecular weights between 14,000 and 82,000. In addition to recognizing these antigens, the convalescent-phase sera detected a protease-sensitive antigen with an apparent molecular weight of 20,000 for one strain and 27,000 for five strains, lipopolysaccharide, and the heat-modifiable proteins. The sera recognized lipopolysaccharide in a serotype-specific manner, whereas their reactions with the heat-modifiable protein were not serotype specific. Convalescent-phase sera recognized components from eight meningococcal serogroups. The concentrations of immunoglobulin G directed to capsular polysaccharide were determined by the enzyme-linked immunosorbent assay; seven acute-phase sera had less than 0.39 μg of antibody per ml, whereas the average concentration in convalescent-phase sera was 3.22 μg/ml and the range was 0.40 to 7.50 μg/ml.

Workers in several laboratories have reported on studies of the human immune response to the capsular polysaccharide of serogroup A and C meningococci in vaccine recipients (1, 7, 8, 11, 19, 23) and in paired acute- and convalescent-phase sera from patients with meningitis (9, 13, 18). Mäkëla et al. determined that a minimum of 2.0 μg of specific antibody per ml is needed for protection from disease (22). Despite the relative homogeneity in noncapsular cell surface antigens of serogroup A meningococci compared with the other serogroups (25), Zollinger and Mandrell outlined a serogroup A lipopolysaccharide (LPS) serotyping system which could be defined by three prototype strains and a heat-modifiable protein system which required three additional strains (30, 31). Presently, very little is known about the human immune response to these and other noncapsular antigens. In this study, the six serotyping strains were used to define the responses to noncapsular antigens in patients from two outbreaks of serogroup A meningococcal disease. The antigens which elicited responses in the acute and convalescent phases of meningitis were visualized by Western blot analyses. In addition, the immunoglobulin G (IgG) antibodies to capsular polysaccharide were quantitated by an enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Antisera and meningococci. Human sera were collected from 14 patients who were diagnosed by culture analysis as having serogroup A meningococcal meningitis (Table 1) by N. Girgis (Naval Medical Research Unit 3, Cairo, Egypt). The sera were classified as acute if they were taken up to 4 days after the onset of symptoms and as convalescent if they were taken 7 days or more after onset. Both acute- and convalescent-phase sera were collected from 10 patients, and convalescent-phase sera were obtained from 4 additional patients. Multiple samples of convalescent-phase sera were taken from seven patients. Serogroup A meningococcal prototype strains (serotypes are indicated in parentheses) 120 (L9), 7880 (L10, P20), 7889 (L11, P22), 7851 (P21), 139 (P19), and 106 (P23) were isolated from patients with disseminated meningococcal disease (31) and were supplied by W. Zollinger (Walter Reed Army Institute of Research, Washington, D.C.). The strains were not selected for any colony morphology characteristics. Several bacterial lysates were prepared (see below) and stored frozen to avoid continued passage of the strains. Strains from other serogroups of meningococci were obtained from the collection of N. Vedros (University of California, Berkeley).

Western blot assay. The bacterial strains were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer, and the antigenic components were separated on a 10 to 15% SDS–polyacrylamide gradient slab gel as previously described (21, 26). After electrophoresis, the material resolved in the gel was electrophoretically transferred (27) to nitrocellulose paper, and a Western blot assay (3) was performed as previously described (26), with the following modifications. The nitrocellulose papers were immersed in a blocking buffer (phosphate-buffered saline [PBS] containing 10% fraction V bovine serum albumin) for 1.5 h at 37°C and then in filtered human antisera diluted 1:100 in blocking buffer for 1.5 h at room temperature. The papers were washed with four changes of PBS at room temperature (15 min/wash). They were then shaken in *Staphylococcus aureus* protein A-peroxidase conjugate (Sigma Chemical Co., St. Louis, Mo.) at a dilution of 1:1,000 in blocking buffer for 1 h at room temperature and washed in PBS as described above. The antigen-antibody complexes were visualized by adding the substrate 3,3'-diaminobenzidine (Sigma). The color development was stopped by washing the nitrocellulose in distilled water. Lanes containing molecular weight markers (Sigma) were stained separately in 0.1% amido black in 10% acetic

† Present address: IGEN, Inc., Rockville, MD 20852.

TABLE 1. ELISA for human IgG directed to polysaccharide

Patient no.	Onset date	Age (yr)	Sex[a]	Type of sera[b]	Days past onset	Antibody concn (μg/ml) to polysaccharide[c]
885	28 March 1970	5	F	A	4	<0.39
				C	59	2.69 ± 0.44
875	29 March 1970	15	M	A	1	<0.39
				C_1	6	6.85 ± 0.76
				C_2	13	3.48 ± 0.32
889	1 April 1970	2	M	A	2	<0.39
				C	13	1.90 ± 0.40
867	28 March 1970	10	M	A	1	<0.39
				C_1	5	3.78 ± 0.14
				C_2	12	2.46 ± 0.17
				C_3	19	1.42 ± 0.09
865	25 March 1970	14	M	A	2	<0.39
				C	9	2.84 ± 0.08
876	28 March 1970	7	F	A	2	1.95 ± 0.15
				C	6	0.80 ± 0.08
886	27 March 1970	5	M	A	6	<0.25
				C_1	12	0.40 ± 0.15
				C_2	19	1.0 ± 0.08
4509	12 March 1981	21	M	A	3	<0.39
				C	17	4.64 ± 0.30
4385	29 January 1981	21	M	A	3	3.57 ± 0.19
				C	41	5.30 ± 0.76
4484	7 March 1981	21	M	A	2	5.96 ± 0.71
				C	12	2.56 ± 0.26
864	22 March 1970	5	M	C_1	9	5.51 ± 0.27
				C_2	15	2.56 ± 0.36
				C_3	22	1.87 ± 0.28
4149	7 May 1980	23	M	C_1	11	1.93 ± 0.16
				C_2	15	2.68 ± 0.31
4387	29 January 1981	21	M	C_1	5	3.35 ± 0.64
				C_2	13	5.08 ± 1.15
				C_3	41	3.28 ± 1.51
4358	3 January 1981	53	M	C_1	5	1.25 ± 0.55
				C_2	11	5.26 ± 1.05
				C_3	28	7.50 ± 1.70

[a] M, Male; F, Female.

[b] A, Acute phase; C, convalescent phase (based on the number of days past onset of symptoms or on the Western blot patterns for days 5 and 6).

[c] Mean ± standard deviation for duplicate wells of at least two dilutions, except for IgG values less than 1 μg/ml, where the average of the duplicate wells is given.

acid–45% methanol. To ensure that the protein A conjugate was not adventitiously binding to particular bands, a control was included in which the nitrocellulose was incubated with protein A-peroxidase after blocking. No bands were detected upon addition of the substrate to this control. Control sera from three people in Egypt and the United States also did not react with the meningococci in the immunoblotting analyses.

Bacterial lysates were also prepared for SDS-polyacrylamide gel electrophoresis by incubation at 37°C for 2 h instead of boiling. The 10 to 15% gradient gel was stained with Coomassie blue R-250 (21) or subjected to a Western blot analysis for identification of the heat-modifiable proteins. All six strains contained proteins which exhibited heat modifiability on SDS-polyacrylamide gels and could be identified by Coomassie blue staining. Purified LPS (28) and pili (2) from strain 4402, a laboratory strain with an L10 serotype, were also subjected to Western blot analyses with a convalescent-phase serum as a means of identifying these meningococcal components. The apparent molecular weight of the pili was approximately 16,000, and LPS migrated to the bottom of the gel in a cone-shaped pattern. A monoclonal antibody directed against pili demonstrated that each of the prototype strains is piliated (26).

An outer membrane preparation was made (6) from meningococcal strain 4402. Samples (11 μg of protein) in 25 mM Tris-hydrochloride (pH 6.8)–0.1% SDS were treated with 0.5 or 2.5 μg of *S. aureus* V-8 protease (Miles Laboratories, Inc., Naperville, Ill.) or buffer for 0.5 h at 37°C. The *S. aureus* V-8 protease was denatured by boiling the samples in SDS-polyacrylamide gel sample buffer for 5 min before application to an SDS-polyacrylamide slab gel. The treated and untreated samples were compared by Western blot analysis with convalescent-phase serum from patient 4484.

Quantitative ELISA for human IgG. The standard curves used for the radioimmunoassay described by Zollinger and Boslego (29) were adapted for an ELISA (5) as follows. Duplicate wells in polystyrene plates (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight at 4°C with 0.1 ml of an 18% (wt/vol) sodium sulfate fraction of goat anti-human immunoglobulin serum (Cappel Laboratories, Malvern, Pa.) at a concentration of 10 μg/ml in 0.06 M carbonate buffer (pH 9.6). The plates were washed in high-salt PBS (0.01 M phosphate buffer [pH 7.4], 0.35 M sodium chloride) containing 0.05% Tween 20 and 1 mg of bovine serum albumin (Sigma) per ml and then blocked by incubation for 4 h at 37°C with PBS and Tween containing 1% bovine serum albumin. The standard solutions (0.1 ml containing between 2 and 2,000 ng of polyclonal human IgG [Calbiochem-Behring, San Diego, Calif.] per ml of blocking buffer) were added to duplicate wells and incubated overnight at 4°C. The plates were washed three times as described above, and 0.1 ml of blocking buffer containing 0.2 μg of mouse monoclonal anti-human IgG (Cappel) per ml was added to each of the wells. After 2 h at 37°C the plates were washed, and 0.1 ml of goat anti-mouse immunoglobulin-alkaline phosphatase conjugate (Sigma) at a dilution of 1:1,000 in blocking buffer was added to each well. The plates were incubated for 1 h at 37°C and washed, and 0.1 mg of phosphatase substrate (Sigma) in 0.1 ml of diethanolamine buffer (10% diethanolamine, 0.1 mg of magnesium chloride per ml, 0.02% sodium azide, pH 9.8) was added to each well. After incubation at room temperature, the plates were read at a wavelength of 405 nm with a Multiskan spectrophotometer (Flow Laboratories, Inc., McLean, Va.).

Each plate contained the IgG standards to eliminate plate-to-plate variation, and the other wells in the plate were coated with antigen by adding 0.1-ml portions of solutions containing 10 μg of poly-L-lysine hydrobromide (Sigma) per ml of PBS for 2 h at 37°C, followed by 10 μg of polysaccharide vaccine A (Institut Merieux, Lyon, France) per ml of carbonate buffer incubated overnight at 4°C. At the same time that the IgG standards were added, serial dilutions of human serum samples diluted from 1:100 to 1:1,600 in blocking buffer were added to duplicate antigen-coated wells. All washing and subsequent incubation steps were performed as described above. Since the immunoglobulins, at low dilutions, compete for the antigen, each serum was assayed at multiple dilutions to ensure that the antibody concentrations corresponded with the dilutions. The standard curves were fitted by an iterative Marquardt nonlinear least-squares program, and the antibody concentrations were determined by interpolation and linear least-squares regression with a Commodore PET computer, using programs adapted from those written for the Wang 2200 computer (4).

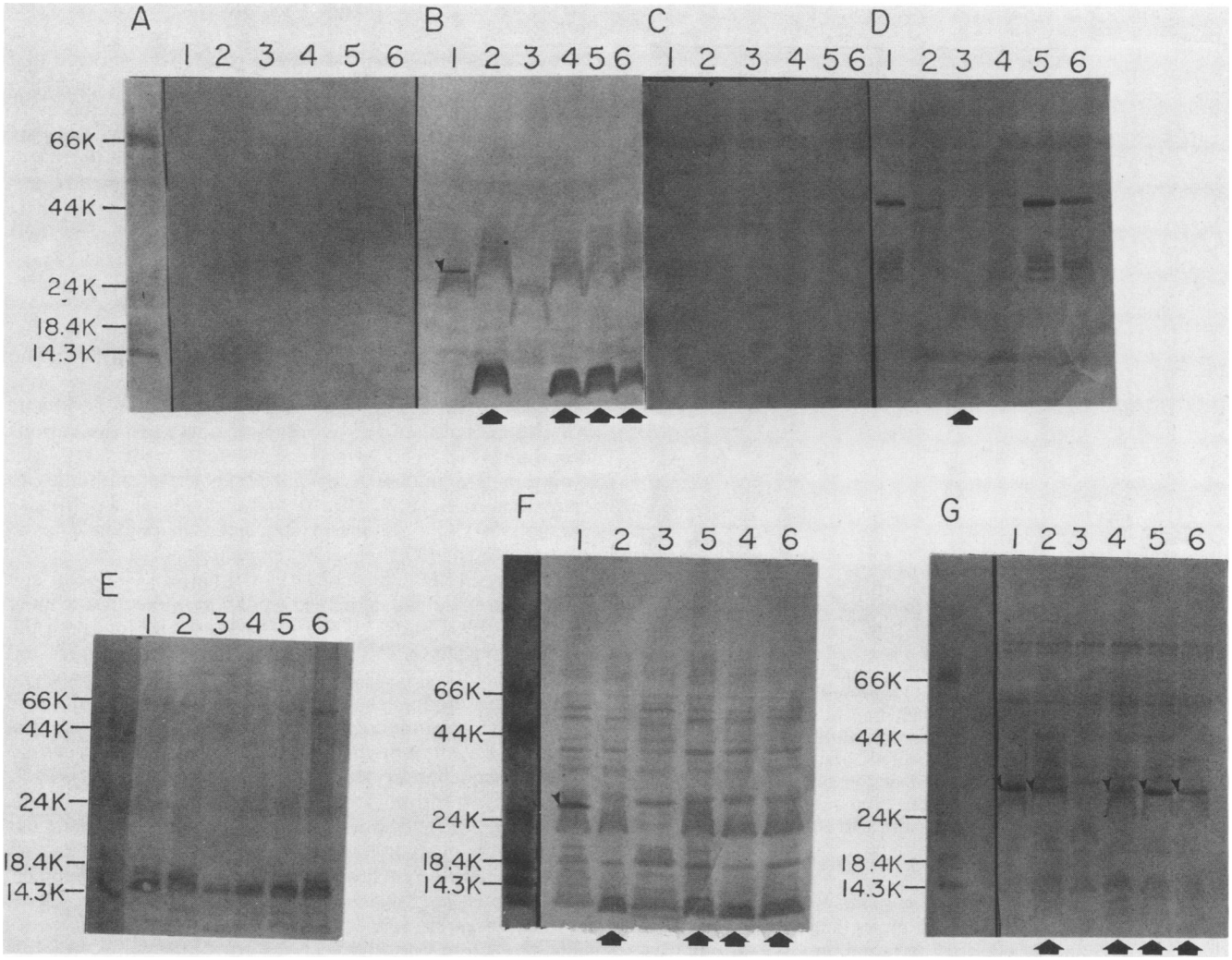

FIG. 1. Western blot analyses for the following human sera: patient 889 acute phase (A), patient 889 convalescent phase (B), patient 875 acute phase (C), patient 875 convalescent phase 2 (D), patient 4484 acute phase (E), patient 4484 convalescent phase (F), and patient 864 convalescent phase 3 (G). The prototype strains (strains 120, 7880, and 7889) for serotyping serogroup A meningococci by LPS were placed in lanes 1 through 3, respectively. The strains for serotyping meningococci based upon the heat-modifiable protein (strains 7880, 7889, 7851, 139, and 106) were placed in lanes 2 through 6, respectively. The LPS (arrows), heat-modifiable protein (arrowheads), and molecular weight markers are indicated for each serum tested. 66K, Molecular weight, 66,000.

RESULTS

All six serotyping prototype strains for serogroup A meningococci exhibited the outer membrane protein banding patterns on SDS-polyacrylamide gels described by Zollinger and Mandrell (31), with the major bands appearing at molecular weights of approximately 45,000, 40,000, and 35,000 and the heat-modifiable protein appearing at a molecular weight of 28,000. Additional heavily stained bands also appeared at molecular weights of 16,000, 64,000, and 76,000.

Western blot analyses with acute-phase sera. Of the 10 acute-phase sera, 4 did not react with meningococcal lysates (Fig. 1A), but 6 sera recognized antigens in at least one of the following molecular weight regions: 14,000 to 18,000, 25,000 to 27,000, 45,000, 55,000, 64,000, and 76,000 to 82,000 (Fig. 1C). Three acute-phase sera (from patients 876, 4385, and 4484) yielded intense staining patterns (Fig. 1E) compared with the other acute-phase sera.

Western blot analyses with convalescent-phase sera. The convalescent-phase sera from 14 patients yielded variable staining patterns when they were subjected to Western blot analyses. Lower-molecular-weight antigens (14,000 to 18,000), one of the outer membrane proteins (molecular weight, 45,000), and the other higher-molecular-weight antigens recognized by the acute-phase sera were also visualized by the convalescent-phase sera (Fig. 1D, F, and G).

LPS bands were visualized by convalescent-phase sera in a serotype-specific manner. Two of the three LPS serotypes were detected; serotype L10 (strains 7880, 7851, 139, and 106) was recognized by 12 of the sera (Fig. 1B, F, and G), and serotype L11 (strain 7889) was recognized by two sera (sera from patients 875 and 876) (Fig. 1D).

A heat-modifiable protein in strain 120 was stained intensely by six convalescent-phase sera (Fig. 1B, F, and G); this protein was stained in strain 139 (P19) by three of the six convalescent-phase sera mentioned above and in strains 7880 (P20), 7851 (P21), 139 (P19), and 106 (P23) by one of the six sera (Fig. 1G). This antigen was identified by Western blot analysis; the apparent molecular weight shifted from 20,000 at 37°C to 28,000 at 100°C (Fig. 2).

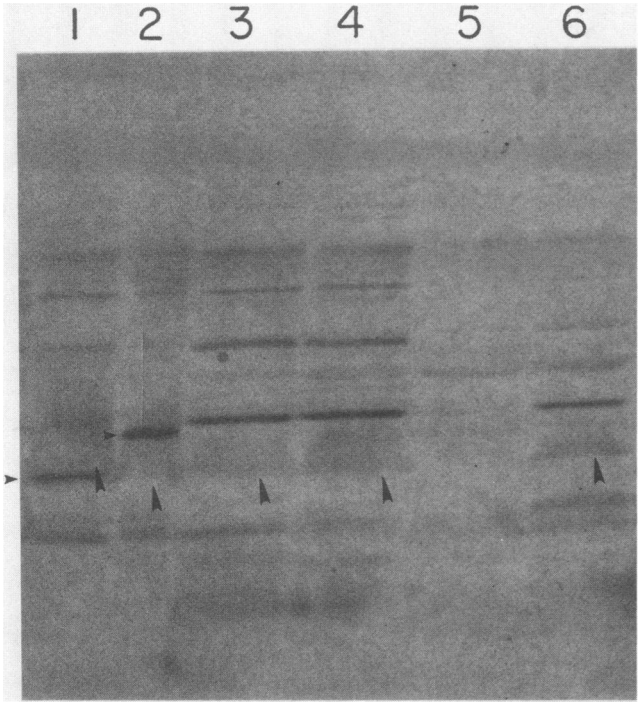

FIG. 2. Characterization of the cone-shaped antigen by Western blot analysis. Meningococci (strain 120) lysed at 37°C (lane 1) or 100°C (lane 2) and outer membrane preparations of strain 4402 treated with buffer at 37°C (lane 3) and 100°C (lane 4) or with 2.5 μg (lane 5) or 0.5 μg of *S. aureus* V-8 protease (lane 6) were subjected to a Western blot analysis with human convalescent-phase serum from patient 4484. The heat-modifiable protein and the cone-shaped antigen are indicated by small and large arrowheads, respectively.

An antigen which had a molecular weight of approximately 20,000 (based on protein molecular weight markers) for strain 7889 and 27,000 for the remaining five strains was detected by Western blot analysis with convalescent-phase sera (Fig. 1B, D, F, and G) but not by Coomassie blue staining. The reaction was cone shaped on the immunoblots. To determine whether this antigen was a protein or partly comprised of protein, an *S. aureus* V-8 protease digest of an outer membrane preparation was subjected to Western blot analysis. Treatment with 0.5 μg of the protease reduced the amount of the antigen that was detected on the blots, whereas no detectable antigen remained at the higher concentration (Fig. 2). This antigen was not heat modifiable (Fig. 2).

Serogroup cross-reactivity. Strains from the other meningococcal serogroups were also tested for the presence of cross-reactive antigens. Each of the three convalescent-phase sera tested reacted with strains belonging to serogroups B, C, W135, X, Y, Z, and 29E. Although there was a variation in the pattern of reactive antigens for each organism and each antiserum, the antigens common to all of the organisms had apparent molecular weights of 78,000 to 82,000, 64,000, 54,000, 28,000, 24,000, and 17,000 (Fig. 3).

ELISA quantitation of IgG. The concentration of IgG directed against the capsular polysaccharide for each serum sample was measured by an ELISA (Table 1). The average concentration of convalescent-phase IgG bound to the antigen was 3.22 μg/ml, and the range was 0.40 to 7.50 μg/ml. In 8 of 10 patients the concentration of IgG was higher in the convalescent-phase serum than in the acute-phase serum.

DISCUSSION

The Western blot analyses of the paired sera showed that there were differences in the acute- and convalescent-phase serum reactions; the convalescent-phase sera reacted more intensely than the acute-phase sera and reacted with the six laboratory strains in distinct patterns. Based upon the Western blot patterns of 9 acute-phase sera and 20 convalescent-phase sera, 6 sera which were taken 5 to 6 days after onset could be classified as acute or convalescent phase by their immunoblotting patterns alone (Table 1).

The convalescent-phase sera recognized LPS in a serotype-specific manner (Fig. 1B, D, F, and G), whereas multiple heat-modifiable proteins were detected on the blots (Fig. 1G). This is consistent with the results of Griffiss, who showed that each serogroup A strain contained only one serotype of LPS but could contain multiple heat-modifiable protein serotypes (14). Cross-reactions between the various serogroups (Fig. 3) confirmed the previous observations that serogroup B meningococcal carriers possess antibodies to serogroups A, B, and C (10, 16) and that convalescent-phase sera do not show serogroup specificity in response to all protein antigens (32).

One antigen yielded a cone-shaped pattern characteristic of LPS, but yielded a higher apparent molecular weight (between 20,000 and 27,000) on the Western blots with the convalescent-phase serum. Although the shape suggested that this material contained polysaccharide, the antigen appeared to contain protein since *S. aureus* V-8 protease digested it. The immunogenicity and mobility of the material were reminiscent of the heat-modifiable protein of serogroup B meningococci described by Poolman et al. (24). However, heat treatment did not change the migration pattern of this antigen (Fig. 2). The antigen described above may be similar to an antigen located on the surface of pathogenic *Neisseria* strains which has recently been identified (W. D. Zollinger, J. S. Ray, E. E. Moran, and R. Seid, Abstr. Int. Conf. Pathol. *Neisseria*, 1984, 58). This antigen also gives cone-shaped reaction patterns on Western blots with convalescent-phase sera, is not heat-modifiable, and does not stain with Coomassie blue.

The reactions of the antisera with the major 35,000- and 40,000-molecular-weight outer membrane proteins were weak or absent on the blot; Poolman et al. (24) also reported the absence of such reactions for meningococcal serogroup B and speculated that this was due to the denaturation of the antigenic site by SDS.

In contrast to the direct detection of serogroup C polysaccharide in SDS-polyacrylamide gels by antisera (24), reactions of serogroup A capsular polysaccharide and antisera were not observed by Western blot analyses. However, polysaccharide-directed IgG antibodies were detected by the ELISA in the sera of these patients (Table 1). A substantial increase in these antibodies was observed in 8 of the 10 patients from whom paired sera were available, a finding consistent with previous reports on the immune response of patients to *Haemophilus* polysaccharide vaccine (20) and serogroup A bacteremic disease (18). Three acute-phase sera showed amounts of antibody which should have been protective (22) and yielded intensely stained Western blots (Fig. 1E). Perhaps the patients were in an early stage of convalescence or the antibody was blocked by high levels of IgA (15). In either case, similar high titers were also noted in 16.2% of the acute-phase cases studied by Käyhty et al. (18).

In another report, human convalescent-phase sera contained an average of 21.7 μg of total antibody to the capsular

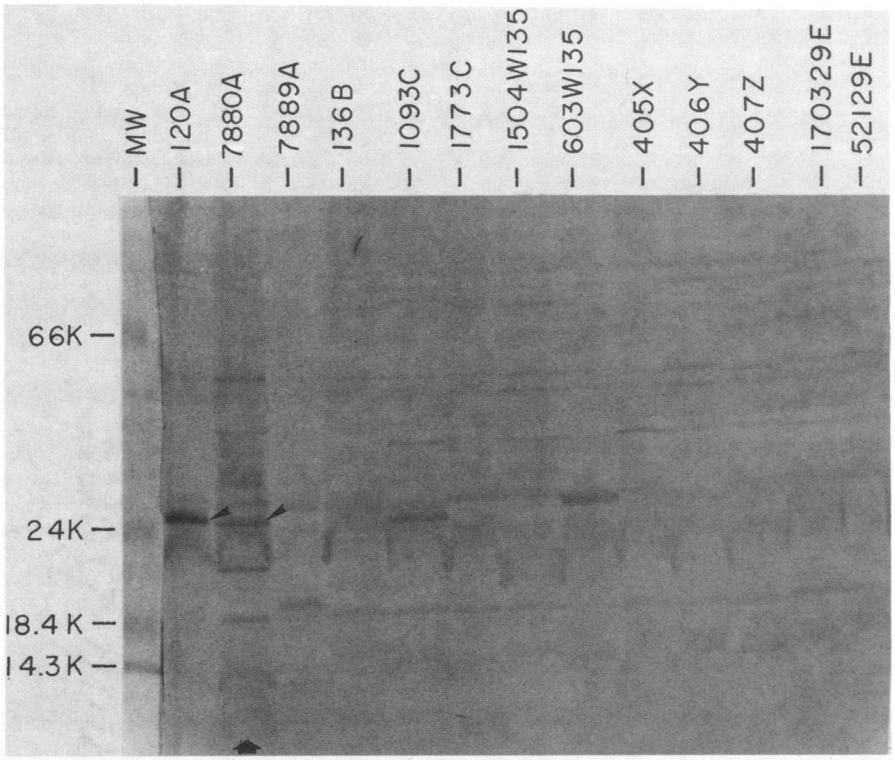

FIG. 3. Western blot reaction of human convalescent-phase serum 2 from patient 864 with meningococcal serogroups. The serogroup A meningococcal LPS (arrow), heat-modifiable protein (arrowheads), and molecular weight (MW) markers are indicated. 66K, Molecular weight, 66,000.

polysaccharide of serogroup A meningococci per ml (18), and between 25 and 44% of these antisera was estimated to be IgG (in other words, between 5.4 and 9.5 μg of IgG per ml). The values calculated in the ELISA were lower and apparently not due to any differences in sensitivity between the ELISA and the radioimmunoassay since both detected approximately 5 ng of IgG per ml. As discussed by Zollinger and Boslego (29), the way in which the polysaccharide antigens are prepared can make as much as a 30% difference in the binding of the antibodies. Since polysaccharide preparations vary in molecular weight (12) and previous calculations of antibody concentrations (13, 17, 19, 23) used high-molecular-weight preparations, perhaps the material used in these studies was not optimal for determining equivalent antibody concentrations.

Gotschlich et al. reported that the IgG antibody concentration should remain stable for 5 months (11). In this study, the anti-capsular IgG levels of two patients (patients 867 and 875) decreased more than the confidence limits between two consecutive convalescent-phase time intervals. One explanation for this discrepancy is improper preparation and storage of the serum samples. These sera contained varying amounts of precipitate, which was removed by filtration but which may have resulted in removal of the antibody. In all cases the drop in polysaccharide-directed antibody level correlated with the intensity of the staining on the Western blots.

Although the antigens recognized by IgM were not elucidated by Western blot analysis or the ELISA, previous work showed that IgM titers against serogroup A polysaccharide approximated the IgG titers at 6 or 7 days (9). Also, the IgM present in the convalescent-phase serum of a patient with serogroup C systemic disease recognized the protein, lipopolysaccharide, and polysaccharide components of serogroup C meningococci (32).

In general, the Western blot acute- and convalescent-phase serum patterns correlated with the ELISA data. The immunoblotting technique could be used for epidemiological studies by allowing the LPS serotype of the infecting organism to be determined without direct isolation of the organism or the LPS.

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