Enzyme-Linked Immunosorbent Assay with a Monoclonal Antibody for Detecting Group A Meningococcal Antigens in Cerebrospinal Fluid

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Hybridomas were produced from spleen cells of BALB/c mice immunized with a membrane preparation from Neisseria meningitidis group A strain 4402 and S194/5.XXOB4.14 myeloma cells. The hybridomas were screened for secretion of antibodies suitable for an enzyme-linked immunosorbent assay (ELISA) diagnostic for group A meningococcal meningitis. One hybridoma antibody, 3G7, was directed against the pilus protein. This antibody bound to all six lipopolysaccharide and protein A meningococcal serotyping strains, as well as to meningococcal strains from serogroups C, W135, and Y, but not to a strain of Escherichia coli, Haemophilus influenzae type b, or to two or more strains of Streptococcus pneumoniae, Neisseria gonorrhoeae, and Salmonella typhi. The ELISA used an antibody, antigen, antibody-conjugate sandwich. Rabbit anti-meningococcal serum was the coating antibody for the antibody sandwich, cerebrospinal fluids contained the bacterial antigens, and 3G7-alkaline phosphatase conjugate was the detecting antibody. The monoclonal antibody conjugate ELISA system was able to detect group A meningococcal antigens in 21 of 25 cerebrospinal fluid specimens that were positive in an immune rabbit serum conjugate ELISA; cerebrospinal fluid samples from patients with Haemophilus meningitis served as the controls. Counterimmunoelectrophoresis detected meningococcal antigens in 16 of the same 25 cerebrospinal fluid samples.

MATERIALS AND METHODS

Bacterial strains and CSF. N. meningitidis group A strain 4402 and CSF samples were obtained from N. Girgis of the Naval Medical Research Unit, no. 3, Cairo, Egypt (18). Serogroup A meningococci were cultured from CSF specimens which were obtained from 25 patients upon admission to the hospital in 1981. Of these specimens, 12 were obtained from male patients between the ages of 20 and 27 years, 3 CSF samples were obtained from female patients between 5 and 18 years old, and the remaining samples were from boys between 2 and 15 years old. CSF samples which yielded Haemophilus influenzae upon culture or contained detectable antigen were used as controls. All CSF specimens were stored frozen at −20°C. The organisms used in this study are listed in Table 1. The meningococci, H. influenzae type b, and Streptococcus pneumoniae were grown on either blood or chocolate agar plates (Microbiological Media, San Ramon, Calif.), Salmonella typhi was grown on MacConkey plates, and Escherichia coli was grown in Luria broth (Difco Laboratories, Detroit, Mich.). All organisms were grown at 37°C.

Production of hybridomas. An outer-membrane serotype antigen preparation (9) was made from N. meningitidis 4402. BALB/c mice (8 weeks old) were injected intraperitoneally with 10 μg of the serotype antigen and boosted after 1.5, 4, and 12 weeks. Spleen cells were harvested 3 days after the last injection and fused with S194/5.XXOB4.14 myeloma cells (provided by I. Trowbridge, The Salk Institute for Biological Studies, La Jolla, Calif.), essentially as outlined by Fazekas de St. Groth and Scheidegger (8). The spleen and myeloma cells were mixed at a ratio of 2:5 and fused with 50% (wt/wt) polyethylene glycol 4000 (gas-chromatography grade, E. Merck AG, Darmstadt, Federal Republic of Germany) in 5% dimethyl sulfoxide and water. The fused cells

Meningococcal meningitis, typically diagnosed in clinical laboratories by culture, can also be diagnosed by immunological detection of antigens in cerebrospinal fluid (CSF) specimens. For example, the capsular polysaccharides of Neisseria meningitidis can be detected in CSF by counterimmunoelectrophoresis (CIE) (6, 11, 18), coagglutination (17), and latex agglutination (15, 23). The agglutination assays are commercially available for A and C meningococcal groups. Radioimmunoassays have also been used (12), but some isotopes have relatively short half-lives and waste disposal can be costly. Meningococcal antigens have also been detected in CSF and serum samples by enzyme-linked immunosorbent assay (ELISA) (20) which has proven to be more sensitive than coagglutination, CIE, and latex agglutination for detection of polysaccharide (C. M. Prato, J. E. Sippel, W. R. Sanborn, and N. I. Girgis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C182, p. 342).

The advantages of an ELISA are many and have been reviewed by Yolkken (24). However, like all immunoassays, an effective ELISA system requires immunoglobulins that are specific and have adequate affinities for the antigenic sites. Hyperimmune serum contains antibodies to many determinants whose characteristics vary from batch to batch. Monoclonal antibodies therefore could improve the ELISA by increasing specificity and by providing consistency in diagnostic reagents.

In this study, an ELISA was developed with a mouse monoclonal antibody which was specific for meningococcal pili and reacted with all the group A serotyping strains, making it suitable as a diagnostic reagent for group A meningococcal meningitis. This ELISA was compared with CIE and an ELISA which used rabbit immune serum.

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were diluted in Iscove modified Dulbecco medium ( Gibco Laboratories, Grand Island, N.Y.) containing 5 x 10^5 M 2-mercaptoethanol, 1 mg of kanamycin sulfate (Gibco Laboratories) per ml, 20% fetal calf serum, 10^-4 M hypoxanthine, 8 x 10^-1 M aminopterin, and 1.6 x 10^-5 M thymidine (13). One-half of the medium was previously conditioned by culture of mouse peritoneal macrophages. The cells were delivered into 96-well plates at a density of 7 x 10^4 total cells per well. Surviving hybridomas grew in 408 out of 2,592 wells; by the analysis of Coller and Coller, the likelihood that these cultures were monoclonal was 92% (4). Of the 408 hybridomas, 156 were positive by ELISA (see below) for antibody directed against the whole organism in the first screen after 2 weeks. After 2 months only five of these continued to be stable. Hybridomas of interest were recloned once by limiting dilution. Ascites fluids were produced by the intraperitoneal injection of 10^7 cells into BALB/c mice primed with 0.5 ml of pristane (Aldrich Chemical Co., Milwaukee, Wis.).

ELISA. The hybridoma supernatant fluids were assayed by the ELISA technique (7, 20). Wells in poly styrene Immulon 1 plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 0.1 ml of 0.06 M carbonate buffer (pH 9.6) containing 10^-7 Formalin-fixed bacteria overnight at 4°C. The plates were then washed three times in phosphate-buffered saline containing 0.05% Tween-20 (PBS-Tween), the hybridoma supernatant fluids (0.1 ml) were added, and the plates were incubated at 37°C for 1 h. After washing as above, 0.1 ml of goat anti-mouse immunoglobulin-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) was added at a 1:1,000 dilution in PBS-Tween. The plates were incubated 1 h at 37°C and washed again, and 0.1 ml of phosphatase substrate (p-nitrophenyl phosphate; Sigma) was added. The absorbance at 450 nm (A450) was read in a Multiskan reader (Flow Laboratories, Inc., Hamden, Conn.) after incubation at room temperature.

Rabbit antimingenococcus (N. Vedros, Naval Biosciences Laboratory, Oakland, Calif.), rabbit antiguonococcus (G. F. Brooks, University of California, San Francisco, Calif.), rabbit anti-Haemophilus (Hyland Diagnostics, Deerfield, Ill.), and rabbit antipneumococcus (Statens Serum Institute, Copenhagen, Denmark) sera were also tested by ELISA for binding to wells coated with various bacteria. Goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Miles Laboratories, Inc., Elkhart, Ind.) was used at 1:1,000 dilution to detect the rabbit immunoglobulins.

Monoclonal antibody isotyping. Isotypes of the monoclonal antibodies were determined by ELISA; the hybridoma supernatant fluids were diluted 1:10 in the carbonate coating buffer and used to coat the microtiter plates. Dilutions (1:1,000) of rabbit antisera directed against mouse immunoglobulins M, G1, G2a, G2b, and G3 (Bio-Research Laboratories, Charleston, S.C.) were added to the washed plate. After a 1-h incubation (37°C), the plates were washed, 0.1 ml of goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate was added at a 1:1,000 dilution, and the assay was completed as above.

**Antibody specificity assay.** Molecular weight markers and suspensions of live bacterial strains were boiled in sodium dodecyl sulfate-polyacrylamide gel sample buffer (14) for 5 min before application to a 10 to 15% polyacrylamide gradient slab gel. After electrophoresis, the material resolved in the gel was electrophoretically transferred (100 mA for 18 h) to nitrocellulose paper in a Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, Calif.), and an immunoperoxidase assay was performed (3, 21) with the following modifications. The nitrocellulose papers were immersed and washed in blocking buffer and washed in PBS as before. The hybridoma supernatant fluids were heat inactivated at 56°C for 30 min or unheated and incubated with nitrocellulose paper in Trans-Blot apparatus. After incubation at room temperature for 1 h, the nitrocellulose papers were immersed and washed in PBS as above. The nitrocellulose papers were then incubated with goat anti-mouse immunoglobulin-alkaline phosphatase conjugate (Cappel Laboratories, Westchester, Pa.) at a 1:1,000 dilution in blocking buffer and washed in PBS as above, and the antigen-antibody complexes were visualized by addition of a substrate solution consisting of 0.3 mg of

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**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pili^a</th>
<th>Serotype^b</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>N. meningitidis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4402-A</td>
<td>+</td>
<td>L10 (LPS)</td>
<td>c</td>
</tr>
<tr>
<td>120-A</td>
<td>+</td>
<td>L9 (LPS)</td>
<td>d</td>
</tr>
<tr>
<td>7880-A</td>
<td>+</td>
<td>L10, P20 (LPS, P)</td>
<td>d</td>
</tr>
<tr>
<td>7889-A</td>
<td>+</td>
<td>L11, P22 (LPS, P)</td>
<td>d</td>
</tr>
<tr>
<td>7851-A</td>
<td>+</td>
<td>P21 (P)</td>
<td>d</td>
</tr>
<tr>
<td>139-A</td>
<td>+</td>
<td>P19 (P)</td>
<td>d</td>
</tr>
<tr>
<td>106-A</td>
<td>+</td>
<td>P23 (P)</td>
<td>d</td>
</tr>
<tr>
<td>981-A</td>
<td>+</td>
<td>L9 (LPS)</td>
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</tr>
<tr>
<td>136-B</td>
<td>+</td>
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<td>d</td>
</tr>
<tr>
<td>1773-C</td>
<td>+</td>
<td>L10 (LPS)</td>
<td>c</td>
</tr>
<tr>
<td>1093-C</td>
<td>+</td>
<td>P23 (P)</td>
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<tr>
<td>521-29E</td>
<td>+</td>
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<tr>
<td>603-W135</td>
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<td>GC 3 (LPS)</td>
<td>f</td>
</tr>
<tr>
<td>1340-Z</td>
<td>+</td>
<td>GC 3 (LPS)</td>
<td>f</td>
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<tr>
<td>407-Z</td>
<td>+</td>
<td>GC 3 (LPS)</td>
<td>f</td>
</tr>
<tr>
<td>1614-Y</td>
<td>+</td>
<td>GC 3 (LPS)</td>
<td>f</td>
</tr>
<tr>
<td>406-Y</td>
<td>+</td>
<td>GC 3 (LPS)</td>
<td>f</td>
</tr>
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</table>

^a Piliation status: +, has pili; -, does not have pili; if unmarked, unknown.

^b Antigen used for typing is within parentheses; LPS, lipopolysaccharide; P, protein; and PS, capsule polysaccharide.

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G. F. Brooks, University of California, San Francisco, Calif.

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**R. vibrio**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pili</th>
<th>Serotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>372 Vi</td>
<td>+</td>
<td>L9 (LPS)</td>
<td>c</td>
</tr>
<tr>
<td>292 Vi</td>
<td>+</td>
<td>L9 (LPS)</td>
<td>c</td>
</tr>
</tbody>
</table>

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* W. Zollinger, Walter Reed Army Institute of Research, Washington, D.C.
* N. Vedros, Naval Biosciences Laboratory, University of California, Oakland, Calif.
* G. F. Brooks, University of California, San Francisco, Calif.
3,3'-diaminobenzidine (Sigma) per ml and 0.005% hydrogen peroxide in 0.05 M Tris-hydrochloride buffer (pH 7.4). The molecular marker lanes were not subjected to the immunoperoxidase staining protocol but were stained separately with 0.1% amido black in 10% acetic acid and 45% methanol.

Assays for bacterial antigens in CSF. Immunoglobulin fractions were precipitated from rabbit antiserum directed against group A meningococci or hybridoma-induced mouse ascitic fluid by adding ammonium sulfate to a concentration of 50% (22). The precipitate was resolubilized and dialyzed against PBS. The protein concentration was determined (16), and a 2-ml sample was conjugated to alkaline phosphatase type VII-S (Sigma) by glutaraldehyde fixation (22). A checkerboard ELISA was performed to determine the optimum dilution of the conjugates; meningococcal strain 4402 was the coating antigen, and the conjugates were diluted from 1:200 to 1:2,000.

The ELISA for bacterial antigens was performed with microtiter plates coated with 0.1 ml of carbonate buffer containing 5.3 μg of rabbit antiserum overnight at 4°C. After washing and blocking (1 h at 37°C) with PBS-Tween containing 1 mg of bovine serum albumin per ml, 0.1-ml samples of CSF or meningococcal strain 4402 were added to duplicate wells and incubated for 1 h at 37°C. The plates were washed, and monoclonal or rabbit antibodies conjugated to alkaline phosphatase were added to the wells at a 1:400 and 1:1,000 dilution, respectively, as determined by the checkerboard assay. Incubation was continued for 1 h at 37°C, the plates were washed again, substrate was added, and the color was developed at room temperature.

CIE was performed on the CSF specimens (11) with rabbit hyperimmune antiserum, with group A polysaccharide vaccine (Merieux Institute, Lyon, France) as a positive control and CSF samples known not to contain bacterial antigens as the negative controls.

Pili purification. N. meningitidis 4402 was grown on chocolate agar plates, transferred to Trypticase soy broth, and grown at 37°C for 22 h. The organisms were centrifuged, and the pili were solubilized in 0.15 M ethanolamine, pH 10.5 (1). After centrifugation (17,000 × g for 15 min), the supernatant fluids were precipitated by addition of ammonium sulfate to 10% saturation. The subsequent pellet was resuspended and dialyzed in 75 mM Tris-hydrochloride buffer (pH 7.4) before the protein concentration was determined. A sample of 1.5 μg of pili was applied to a 10 to 15% sodium dodecyl sulfate-polyacrylamide gel for an immunoblotting assay.

RESULTS

Production of hybridomas. Spleen cells of BALB/c mice immunized with an outer-membrane preparation of N. meningitidis group A strain 4402 were fused with S194/5.XXOBU.14 myeloma cells to produce hybridomas. The hybridoma supernatant fluids were assayed for antibody by an ELISA which used strain 4402 as the antigen. Colonies which secreted antibody directed against strain 4402 were cloned and assayed were produced.

Specificity. The specificity of the monoclonal antibodies was determined by a Western blot analysis. One of the monoclonal antibodies designated 3G7 was of the immunoglobulin G2a subclass and bound to a low-molecular-weight component of 16,000. An immunoblot assay with a pili protein preparation showed that 3G7 was specific for the pili protein of the meningococcus (Fig. 1).

FIG. 1. Immunoblot reaction of 3G7 with strain 4402, purified pili, and group A serotyping strains. The bacterial lysates, purified pili, and molecular weight marker components were resolved on a 10 to 15% sodium dodecyl sulfate-polyacrylamide slab gel. The pili subunits of all the group A meningococcal strains (4402-A, 120-A, 7880-A, 7889-A, 7851-A, 106-A, and 139-A) were detected by an immunoperoxidase stain with 3G7.

One of the criteria for choosing a monoclonal antibody suitable for an ELISA was the capability of the antibody to bind all of the group A serotyping strains (25). Each of the protein and lipopolysaccharide group A serotyping strains was subjected to a Western blot analysis in which the pili from each of the strains reacted with monoclonal antibody 3G7 (Fig. 1). Some variation in the mobilities of the pili was evident, indicating pili heterogeneity of the group A meningococcus.

The ability to discriminate between meningococci and other organisms, especially those which may also cause meningitis, is an important characteristic required of an antibody suitable for use as a diagnostic reagent. The monoclonal antibody 3G7 did not bind to E. coli, H. influenzae, Neisseria gonorrhoeae F62, S. typhi 372 or 292, or S. pneumoniae 35 by the Western blot analysis (data not shown).

To determine whether this antibody could be used in a broad-spectrum ELISA, the monoclonal antibody 3G7 was also tested by the immunoblot assay for reactivity against strains from the other major meningococcal serogroups. Four strains did contain pili which bound to 3G7 (Fig. 2). The identity of the other reactive lower-molecular-weight protein present in this 4402 lysate was not pursued.

Development of the ELISA. Since the goal was to develop a diagnostically useful ELISA, the organisms screened by the immunoblot technique, a rough nontypable, types 1, 2, and 14 pneumococcal strains, and gonococcal strains 8551 and 4505, were also tested by an ELISA for antibody binding. Rabbit hyperimmune antimeningococcal serum and the mouse monoclonal antibodies were compared by the ELISA, and the results with 11 representative strains are shown in Fig. 3. The monoclonal antibody detected all of the group A meningococcal strains but not the other organisms, whereas the rabbit anti-group A meningococcal serum was less specific and bound to the group A strains as well as to the B and C meningococcal groups. Each of the organisms was detected by its homologous serum (data not shown).

To optimize conditions by reducing background to a minimum, an ELISA was run in which microtiter wells were
coated with monoclonal immunoglobulin purified from ascites or rabbit antiserum. PBS-Tween buffer containing bovine serum albumin was substituted for antigen, and then 3G7 conjugate was added to the plates. The conjugate apparently nonspecifically bound to the wells coated with the monoclonal antibody as evidenced by the developing color, whereas it did not bind to wells coated with rabbit antiserum. Attempts to reduce the background after coating with the monoclonal antibody by blocking with nonimmune rabbit serum were not successful; therefore, rabbit antiserum was used to coat the microtiter plate wells in subsequent tests.

**Detection of bacterial antigens in CSF.** Detection of the meningococcal antigens in CSF by the ELISA was first performed with the rabbit immunoglobulin conjugate ELISA system. The ELISA controls were CSF from which *H. influenzae* type B organisms were detected. The 25 CSF samples in which meningococcal antigens were detected by the rabbit immunoglobulin conjugate were assayed with the monoclonal antibody conjugate. The means and the standard deviations of the *Haemophilus* control specimens and the range of absorbance values determined for the meningococcal specimens of both systems are presented in Table 2. The 3G7 conjugate was able to detect pili in 21 of the 25 CSF specimens assayed. All *Haemophilus* specimens were negative by the criteria outlined in Table 2.

Each of the 25 CSF samples which contained meningococcal antigens was also subjected to a CIE analysis. The CIE assay detected polysaccharide in 16 of 25 CSF specimens. Two of the CSF specimens contained meningococcal antigens which went undetected by the monoclonal antibody ELISA as well as by the CIE assays.

**DISCUSSION**

The group A meningococcus appears to be the most homogeneous meningococcal group with respect to its complement of outer-membrane proteins (19), and they are therefore an excellent model for testing the usefulness of a single monoclonal antibody in a diagnostic assay. This report describes the development of an ELISA for detection of group A meningococcal antigens in CSF with a monoclonal antibody as the tagged immunoglobulin.

The monoclonal antibody used in this assay was directed against the pilus protein. Craven et al. showed that pili were present on bacteria in 96 to 100% of the meningococcal isolates from carriers and patients (5); this observation suggested that our antipilus monoclonal antibody could be used for detection of meningococci in CSF specimens. Although the first 29 amino-terminal amino acids of pilus protein are identical in various gonococcal and meningococcal strains (10), *N. gonorrhoeae* pili appear to be antigenically heterogeneous (2). The pili of the serotyping strains of meningococcal group A are also not identical because differences in electrophoretic migration were observed (Fig. 1). However, the meningococcal pili share an antigenic site which was recognizable by 3G7. The monoclonal antibody also demonstrated that the pili in other meningococcal serogroups may be dissimilar because it reacted with only one of two group C strains and one of two group Y strains.

The monoclonal antibody conjugate ELISA was able to detect the pilus antigen in 21 of 25 CSF specimens that were positive with the rabbit antibody conjugate ELISA. There are several possible explanations why the monoclonal antibody did not detect pili in four of the specimens; the pili may have been degraded by proteases, the antigenic site of the pili may have been inaccessible, the concentration of pili was low, or the pilus antigen may not have been recognized by 3G7. The monoclonal antibody conjugate ELISA was first performed with the rabbit immunoglobulin conjugate ELISA system. The ELISA controls were CSF from which *H. influenzae* type B organisms were detected. The 25 CSF samples in which meningococcal antigens were detected by the rabbit immunoglobulin conjugate were assayed with the monoclonal antibody conjugate. The means and the standard deviations of the *Haemophilus* control specimens and the range of absorbance values determined for the meningococcal specimens of both systems are presented in Table 2. The 3G7 conjugate was able to detect pili in 21 of the 25 CSF specimens assayed. All *Haemophilus* specimens were negative by the criteria outlined in Table 2.

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may have been below the limit of detection, or the pili may not have been recognized by the antibody because it did not share the antigenic determinant with strain 4402 pili. The monospecific antibody was able to detect antigen in 84% of the CSF specimens. This effectiveness is remarkable when one considers the limited amount of antigen available; the immune serum tag should detect any of the major cell surface antigens in the CSF samples (polysaccharide, lipopolysaccharide, outer-membrane proteins, pili), whereas the monoclonal antibody tag can only detect one determinant on the pilus. Also, CIE with rabbit serum tag detected polysaccharide antigen in only 64% of the same samples.

The hybridoma selected in this study provides a consistent monoclonal antibody which has been shown to act as a specific reagent in the ELISA. This antibody has the advantage over hyperimmune sera because it does not react with organisms such as H. influenzae, which is often a problem with other rabbit antiserum preparations (11, 17). Use of a defined mixture of monoclonal antibodies would be likely to make the assay more sensitive and specific. Currently, we are attempting to prepare suitable monoclonal antibodies against nonpilus antigens of group A meningococci.

ACKNOWLEDGMENT
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LITERATURE CITED