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Use of Monoclonal Antibodies in an Enzyme Immunoassay for Rapid Identification of Group B *Streptococcus* Types II and III

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Streptococci belonging to Lancefield group B are frequently recognized as the etiological agents of sepsis and meningitis in young children. Current methods of identifying these organisms have not been universally accepted because of the time and complexity in performing the studies and a lack of reference antisera. We have developed hybrid myeloma (hybridoma) cell lines which secrete large amounts of antibody against types II and III group B streptococci. Antibodies harvested from supernatants react only with the bacterial strain that was used initially to immunize the animals. We have used the hybridoma antibodies in an enzyme immunoassay and have shown it to be a sensitive and reliable technique for typing group B streptococci. The use of hybridoma antibodies in the enzyme immunoassay may permit early detection of group B streptococcal antigen before cultures are visibly positive.

Beta-hemolytic group B *Streptococcus* (GBS) is the most common pathogen identified in septic neonates (3). Among infants infected during the first 5 days of life (early onset disease), the mortality rate may exceed 50% and one third of the children develop central nervous system involvement. Early administration of penicillin, treatment of maternal GBS carriers (25), and in some cases the combined administration of aminoglycoside antibiotics and penicillin or a penicillin derivative (10) have been suggested by some investigators as a way of decreasing disease mortality or morbidity or both. None of the studies recommending prophylactic antimicrobial therapy has prospectively determined the GBS colonization status of the study infants or mothers; however, each of these therapeutic recommendations should be based upon rapid and accurate identification of GBS in culture specimens. Traditional methods of distinguishing GBS from other pathogens have included capillary precipitin tests with HCl-extracted antigens (9), staphylococcal coagglutination (15), CAMP test (1), latex agglutination (15), and counterimmunoelectrophoresis (15). The clinical usefulness of many of these methods is diminished because of the time required to perform the procedures, the complexity of the tests, and a general lack of standard reference antisera. Recent advancements in the technique of somatic cell hybridization by Köhler and Milstein have permitted development of hybrid myeloma cell lines (hybridomas) which secrete antibody of predetermined specificity (7). The cell lines are immortalized and may be grown in large volumes to generate abundant quantities of monospecific antibody. Using this methodology we have developed specific antibodies against type II and type III GBS which have proven sensitive and useful for typing GBS. The use of these antibodies in an enzyme immunoassay (EIA) provides a rapidly performed, specific, and sensitive test to identify these pathogens.

MATERIALS AND METHODS

Bacterial strains. (i) GBS. Reference strains, supplied by Rebecca Lancefield, were as follows: type II, 18RS21/67/2; type III, D136C.

(ii) *S. pneumoniae*. *Streptococcus pneumoniae* type XIV was isolated and identified by Children's Hospital of Philadelphia Microbiology Laboratory.

Mouse plasmacytoma cell line. SP2/O-Ag 14 a murine plasmacytoma cell line was maintained in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). Cell lines were grown in high humidity and 5% CO_2 and routinely screened for mycoplasma contamination. After fusion the hybrids were grown in HY medium (6): Dulbecco-modified Eagle medium, 10% NCTC (Microbiological Associates), 20% fetal calf serum, 0.2 U of bovine insulin per ml, 0.45 mM pyruvate, and 1 mM oxaloacetate.

Preparation of vaccine. Vaccine was prepared as described by Wilkinson (22). The bacterial strains were grown overnight in 200 ml of Todd-Hewitt broth. The cells were packed by centrifugation, and type II bacteria were suspended in 10 ml of 0.2% formalinized saline. For type III vaccine the cells were resuspended in 6 ml of formalinized saline, and the pH of the suspension was lowered to pH 2 with sterile HCl.

Pepsin was added to a concentration of 0.05% and the mixture was incubated for 2 h at 37°C. The cell suspension was neutralized with 1 N NaOH and brought up to 10 ml with additional 0.2% formalinized saline. All vaccines were then refrigerated at 4°C for 4 to 5 days, at which time sterility tests were performed to make sure there were no viable organisms in the inoculum. Vaccines were diluted 1:10 in sterile saline before use.

Immunization. The mice were given three immunizations at weekly intervals. The first injection consisted of 0.5 ml of bacterial suspension in incomplete Freund adjuvant administered intraperitoneally. The second dose (0.5 ml) diluted in saline was given both intraperitoneally and subcutaneously. The final injection (0.2 ml) was given intravenously.

Animals. Female BALB/c mice at least 3 months old were obtained from Ace Animal Suppliers.

Hybridoma production. Cell fusion and cloning were performed by previously published methods (6).

Radioimmunoassay (4). A polyvinyl chloride plate with 96 V-shaped microtiter wells was filled with RPMI 1640 with HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid, 20 mM) and 1% bovine serum albumin. The plates were incubated for 1 h at room temperature, the media were removed, and 2×10^6 bacteria in formalinized saline were placed into each well. The bacteria were pelleted by centrifugation at 2,500 rpm and were resuspended in 50 μl of the antibody to be tested. After a 1-h incubation on melting ice, the plates were centrifuged (2,500 rpm), and all wells were washed with RPMI 1640 and 1% bovine serum albumin. ^{125}I-labeled antimouse immunoglobulin (Fab specific) was diluted in 0.5% bovine serum albumin to 200,000 cpm/100 μl, and 50 μl was added to each well. The bacteria and radiolabeled antisera were incubated for 1 h on ice. The cells were pelleted and washed again, and the pellets were counted in a gamma counter. Duplicate samples were run for all supernatants on three separate occasions. Each supernatant was tested for binding to type II GBS, type III GBS, and type XIV *S. pneumoniae*. Control supernatants were obtained from actively growing cultures of the mouse plasmacytoma line SP2/O-Ag 14.

EIA. A polyvinylchloride plate with 96 V-shaped microtiter wells was filled with 1% gelatin in RPMI 1640 with 20 mM HEPES buffer (pH 7.6) and incubated for 1 h. The gelatin was removed, and the following reagents were added to each well: 2×10^6 bacteria suspended in 50 μl of 0.1% gelatin in RPMI 1640, and 50 μl of the antibody solution to be tested. The bacteria and antibody were incubated for 0.5 h at room temperature and then centrifuged (2,500 rpm) for 5 min. The pellets were washed twice more with 0.1% gelatin in RPMI 1640, at which time 100 μl of peroxidase-labeled anti-mouse immunoglobulin (Cappel, 1:1,000 dilution) was added to each well. The cells and peroxidase-conjugated antisera were incubated for 0.5 h at room temperature, centrifuged, and washed three times with 0.1% gelatin in RPMI 1640. After the last wash, 100 μl of a solution containing 10 mg of ortho-phenylene diamine and 2 μl of 30% H_2O_2 in 10 ml of citrate buffer was added to each well. The color of the well was recorded at the end of a 30-min incubation and graded 0 to 3+. Duplicate samples were run for all supernatants on three separate occasions. Each supernatant was tested for binding to type II GBS, type III GBS, and type XIV *S. pneumoniae*. Control supernatants were obtained from actively growing cultures of the mouse plasmacytoma line SP2/O-Ag 14.

RESULTS

Cell fusion and cloning. Results from both fusions are summarized in Table 1. Eighty-eight wells from the fusion with spleen cells immunized to type III GBS and 46 wells from the fusion with spleen cells immunized to type II GBS contained growing colonies. Thirty-six percent of the type III hybridomas and 13% of type II hybridomas secreted anti-GBS antibody. Five hybridomas secreting antibody against type II GBS and 14 hybridomas secreting antibody against type III GBS were grown into large volumes for further analysis. The homogeneity of each hybridoma was assured by recloning the cells from each positive well. One hundred percent of colonies cloned in agarose plates secreted antibody with specificity identical to the originally isolated hybridoma cell lines.

Binding assays. (i) Radioimmunoassay. Results for both type II and type III hybridoma supernatants are illustrated in Fig. 1 and 2. The bar graphs are representative of duplicate samples from a single assay. Each supernatant was tested on two additional occasions and yielded identical results. Five supernatants which did not bind to the bacteria are illustrated for comparison. Of 14 antibodies selected for reactivity to type III GBS, 10 bound only to type III bacteria. All five anti-type II GBS antibodies specifically bound to type II bacteria. None of the type II or type III antibodies bound to type XIV *S. pneumoniae*.

EIA. Results for all type II and type III hybridoma antibodies are illustrated in Table 2. The results are consistent with those obtained in the radioimmunoassay. All five type II GBS antibodies produced strong color reactions when incubated with type II bacteria and not with type III GBS. Fourteen of fourteen type III GBS antibodies produced strong color reactions when incubated with type III bacteria. Ten of these

TABLE 1. *Summary of cell fusion*

Immunizing GBS strain	No. of wells tested	No. of wells secreting GBS antibody	% of wells secreting antibody against immunizing strain
Type III	88	32	36
Type II	46	6	13

reacted specifically with type III GBS, whereas 4/14 reacted also with type II bacteria. Incubation of type II or type III GBS antibodies with type XIV *S. pneumoniae* did not produce color reaction.

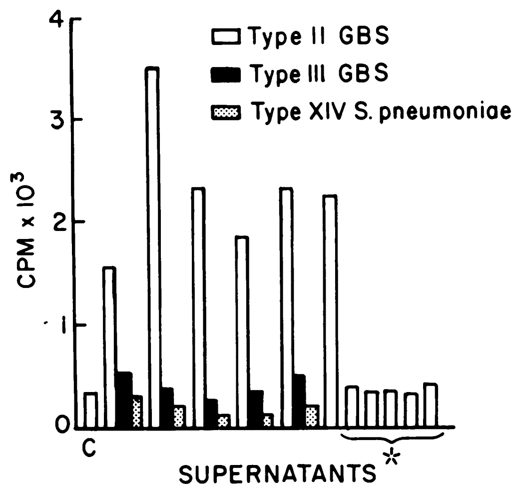

FIG. 1. *Binding of hybridoma supernatants to bacterial cell pellets; mice immunized with type II GBS: radioimmunoassay. Bar graphs are representative of duplicate samples from a single assay. Six supernatants bound to type II GBS. Five samples were grown into large volumes and tested for binding to type XIV S. pneumoniae and type III GBS. Each supernatant was tested on two additional occasions and yielded identical results. Five supernatants which did not bind to the bacteria are illustrated for comparison (last five values on right side). C, Control supernatant; *, supernatants did not bind to type II GBS.*

TABLE 2. *Binding of hybridoma supernatants to bacterial cell pellets: EIA*

Hybridoma supernatant fluid		Bacterial cell pellet		
GBS vaccine	No. tested	GBS type II	GBS type III	*S. pneumoniae* type XIV
Type II	5	5 (3+)[a]	0	0
Type III	14	4 (2+)	14 (3+)	0
None (control)	12	0	0	0

[a] Number (and intensity) of reactants. Intensities recorded as 3+ (strongly positive), 2+ (moderately positive), and 0 (negative).

DISCUSSION

The use of EIA techniques has greatly facilitated the detection and quantitation of specific antibody against viral, fungal, bacterial, or protozooan pathogens and permitted the detection of antigens from these microorganisms in clinical specimens. EIA techniques have been extensively used in the areas of viral diagnosis and epidemiological viral screening. Assays to document antibody titers against rubella (18), cytomegalovirus (19), herpes simplex virus types 1 and 2 (16), and Epstein-Barr virus (20) have already been developed. In most instances, EIA

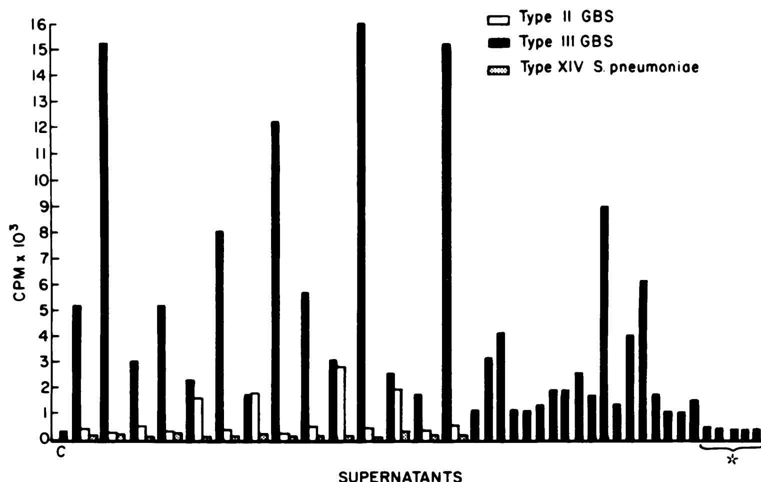

FIG. 2. *Binding of hybridoma supernatant to bacterial cell pellets; mice immunized with type III GBS: radioimmunoassay. Bar graphs are representative of duplicate samples from a single assay. Thirty-two supernatants bound to type III GBS. Fourteen samples were grown into large volumes and tested for binding to type XIV S. pneumoniae and type II GBS. Each supernatant was tested on two additional occasions and yielded identical results. Five supernatants which did not bind to the bacteria are illustrated for comparison (last five values on right side). C, Control supernatant; *, supernatants did not bind to type III GBS.*

has proven severalfold more sensitive than standard serological assays and has been rapidly adapted to both clinical and research laboratories. In addition to its use in antibody detection, EIA has been used clinically to detect bacterial toxins (17) and candida antigen (21) and to identify the antigens of numerous microorganisms in tissue specimens (24). An assay for detection of hepatitis B surface antigen is commercially available (23), and EIAs for herpes simplex virus (11) and hepatitis A are being developed (17).

The major limitation in the development of EIA for bacterial antigens has been in the isolation of high-titer specific antisera. Antisera raised in animals have varied in quality and in the complex background reactivity of the host animal sera (13). Somatic cell hybridization provides the tools to develop highly specific serological reagents that can be produced in large quantities and serve as laboratory standards. Antibody may be harvested from supernatants (microgram-per-milliliter quantities) or tumor-stimulated ascitic fluid (milligram-per-milliliter quantities). Specific antibodies have been produced against viruses (8), haptens (12), soluble proteins (3), and a variety of cell surface antigens (6). The production of monoclonal antibodies to microorganisms will provide a practically unlimited supply of reagents that can clearly define antigenic variations in a given bacterial or virus type and detect antigen in tissue specimens. We have developed hybrid myeloma cell lines which are secreting monoclonal antibodies against type II and type III GBS. The specificity of these antibodies has been demonstrated by the EIA technique and a radioimmunoassay. Ten of fourteen type III hybridoma antibodies and five of five type II hybridoma antibodies reacted only with the corresponding type-specific organism. Additionally, we have demonstrated that the type III antibodies did not bind to type XIV *S. pneumoniae*, suggesting that these hybridoma antibodies are highly specific and directed against the complex neutral buffer-extracted antigen of type III GBS and not the HCl-extracted antigen which is chemically similar to the polysaccharide capsule of type XIV *S. pneumoniae* (2). Using the EIA technique and hybridoma antibodies we have been able to positively type and identify type II and III GBS in just a few hours, and it should be possible to rapidly identify and type any GBS when the appropriate antibodies are made available. This kind of assay can be easily adapted to any microbiology laboratory. Colonies of bacteria can be removed from culture plates, suspended in saline, and pelleted in microtiter plates. Formalin fixation would not be necessary. Results of the EIA can be read automatically with a recently available eight-channel photometer (Multiscan Titertek, Flow Laboratories). Antibodies are now being prepared against two other GBS types (IA and IB) and against the group B-specific antigen. The use of monoclonal antibodies in an EIA may allow detection of antigen in tissue fluid specimens long before cultures are visibly positive, thus permitting early institution of antibiotic therapy in infants with suspected sepsis.

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LITERATURE CITED

1. **Darling, C. L.** 1974. Standardization and evaluation of the CAMP reaction for the prompt presumptive identification of Streptococcus agalactiae (Lancefield group B) in clinical material. J. Clin. Microbiol. **1**:171–174.
2. **Fischer, G. W., G. H. Lowell, M. H. Crumrine, and J. W. Bass.** 1978. Demonstration of opsonic activity and in vivo protection against group B streptococci type III by streptococcus pneumoniae type 14 antisera. J. Exp. Med. **148**:776–786.
3. **Franciosi, R. A., J. D. Knostman, and R. A. Zimmerman.** 1973. Group B streptococcal neonatal and infant infections. J. Pediatr. **82**:707–718.
4. **Hämmerling, G. J., M. Reth, H. Lemke, J. Hewitt, I. Melchers, and K. Rajewsky.** 1977. Fusion of myelomas and T-lymphocytes with immunocytes, p. 551–558. *In* H. Peters (ed.), XXV Coloquium protides of the biologic fluids. Pergamon Press, Oxford.
5. **Kasper, D. L., C. J. Baker, R. S. Baltimore, J. H. Crabbe, G. Schiffman, and H. J. Jennings.** 1979. Immunodeterminant specificity of human immunity to type III group B streptococcus. J. Exp. Med. **149**:327–339.
6. **Kennett, R. H., K. A. Denis, A. Tung, and N. R. Klinman.** 1978. Hybrid plasmacytoma production. Fusions with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. Curr. Top. Microbiol. Immunol. **88**:77–91.
7. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells making antibody of predefined specificity. Nature (London) **256**:495–497.
8. **Koprowski, H., W. Gerhard, T. Wiktor, J. Martinis, M. Shander, and C. M. Croce.** 1978. Anti-viral and anti-tumor antibodies provided by somatic cell hybrids. Curr. Top. Microbiol. Immunol. **88**:8–19.
9. **Lancefield, R. C.** 1933. Serological differentiation of human and other groups of hemolytic streptococci. J. Exp. Med. **57**:571–595.
10. **McCracken, G. H., Jr., and W. E. Feldman.** 1976. Editorial comment. J. Pediatr. **89**:203–204.
11. **Miranda, Q. R., G. D. Bailey, A. S. Fraser, and H. J. Tenoso.** 1977. Solid phase immunoassay for Herpes simplex virus. J. Infect. Dis. (Suppl.) **136**:S304–S310.
12. **Reth, M., G. J. Hammerling, and K. Rajewsky.** 1978. Analysis of the repertoire of anti-NP antibodies in C57BL/6 by cell fusion. Eur. J. Immuno. **8**:393–400.
13. **Siegel, J. D., and G. H. McCracken, Jr.** 1978. Detection of group B streptococcal antigens in body fluids of neonates. J. Pediatr. **93**:491–492.
14. **Steigman, A. J., E. J. Bottone, and B. A. Hanna.** 1975. Does intramuscular penicillin at delivery prevent group B beta hemolytic streptococcal disease of newborn infants? J. Pediatr. **87**:496.

15. **Thirumoorthi, M. C., and A. S. Dajani.** 1979. Comparison of staphylococcal coagglutination and counterimmunoelectrophoresis for bacterial antigen detection. J. Clin. Microbiol. **9**:28–32.
16. **Vestergaard, B. F., P. C. Grauballe, and H. Spangaard.** 1977. Titration of Herpes simplex virus antibodies in human sera by the enzyme linked immunosorbent assay (ELISA). Acta Pathol. Microbiol. Scand. Sect. B **85**:466–468.
17. **Voller, A., A. Bartlett, and D. E. Bidwell.** 1978. Enzyme immunoassays with special reference to ELISA techniques. J. Clin. Pathol. **31**:507–520.
18. **Voller, A., and D. E. Bidwell.** 1975. A simple method for detecting antibodies to Rubella. Br. J. Exp. Pathol. **56**:338–339.
19. **Voller, A., and D. E. Bidwell.** 1975. Enzyme immunoassays for antibodies in measles, cytomegalovirus infections, and after Rubella vaccinations. Br. J. Exp. Pathol. **57**:243–247.
20. **Wallen, W. C., J. M. Mattson, and P. H. Levine.** 1977. Detection of soluble antigen of Epstein Barr virus by the enzyme linked immunosorbent assay. J. Infect. Dis. (Suppl.) **135**:S324–S328.
21. **Warren, R. C., A. Bartless, D. E. Bidwell, M. D. Richardson, A. Voller, and L. O. White.** 1977. Diagnosis of invasive candidasis by enzyme immunoassay of serum antigen. Br. Med. J. **1**:1183–1185.
22. **Wilkinson, H. W.** 1973. Preparation of Group B streptococcus typing antisera. Center for Disease Control, Atlanta, Ga.
23. **Wolters, G., L. Kuipers, J. Kacaki, and A. H. Schuurs.** 1977. Enzyme linked immunosorbent assay for hepatitis B surface antigen. J. Infect. Dis. (Suppl.) **136**:S311–S317.
24. **Yolken, R. H., H. W. Kim, T. Clem, R. G. Wyatt, A. R. Kalica, R. M. Chanock, and A. Z. Kapikian.** 1977. Enzyme linked immunosorbent assay (ELISA) for detection of human neovirus like agent of infantile gastroenteritis. Lancet **ii**:263–266.
25. **Yow, M. D., E. O. Mason, L. J. Leeds, P. K. Thompson, D. J. Clark, and S. Gardner.** 1979. Prevention of intrapartum transmission of group B streptococcal infection by intravenous ampicillin. J. Am. Med. Assoc. **241**:1245–1247.