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Rapid Detection of Group B Streptococcal Antigen by Monoclonal Antibody Sandwich Enzyme Assay

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Group B *Streptococcus* (GBS) is the most common cause of neonatal sepsis and meningitis. Infants at greatest risk to develop invasive disease are delivered to women colonized with GBS in their birth canals and lacking immunity to the colonizing serotype. We have investigated the sensitivity and specificity of a recently developed monoclonal antibody sandwich enzyme immunoassay for detection of GBS antigen. The sandwich enzyme immunoassay detected types II and III GBS at a concentration of 5×10^4 CFU/ml and types Ia and Ib GBS at 5×10^5 CFU/ml. No cross-reactions were noted when each of the GBS serotypes was reacted with antibodies of differing serotype specificities. Type III GBS native antigen was detected at a concentration of 1 ng/ml. The sandwich enzyme assay is more sensitive than other methods currently in use for rapid detection of GBS and is serotype specific. This assay system should prove useful for the detection of GBS colonization during labor and for identification of neonates with invasive disease.

Serious infection in neonates is most frequently associated with group B *Streptococcus* (GBS). Women at greatest risk to deliver infants with early-onset streptococcal disease are heavily colonized with GBS, lack immunity to the colonizing serotype, deliver prematurely, or have GBS bacteremia (1). The incidence of neonatal colonization with GBS in the first day of life varies from 15 to 20% (1), and approximately 1 to 2% of colonized infants will develop invasive disease. When the aforementioned risk factors are present, however, disease frequency increases significantly. Despite improvements in obstetrical and neonatal intensive care, there is 50% mortality for infants with early-onset infections. The administration of prophylactic antibiotics immediately postnatally may not prevent rapid progression of the disease (14). Alternatively, women colonized with GBS may be treated with ampicillin during labor to prevent vertical transmission (17); however, routine culture techniques require 24 to 48 h for processing and culture results are not available until after the infant has been delivered. We have developed a sandwich enzyme immunoassay for the rapid detection of GBS antigen, utilizing monoclonal antibodies (MCAs) for each of the specific serotypes of GBS, and have determined the sensitivity and specificity of this enzyme immunoassay.

MATERIALS AND METHODS

Preparation of vaccine, immunization, and hybridoma production. Vaccines were prepared with formalinized Lancefield reference strains of GBS (type Ia, 090/14; Ib, H36B/60/2; Ic, A909/14; II, 18RS21/67/2; III, D136C). BALB/c mice were given three immunizations of formalinized bacteria at weekly intervals (12,13). The first injection consisted of antigen (0.5 ml) in incomplete Freund adjuvant administered intraperitoneally. The second dose (0.5 ml), diluted in saline, was given intraperitoneally and subcutaneously. The final injection (0.2 ml) was given intravenously. Cell fusion and cloning were performed by previously published methods (11). Hybridomas were tested for binding to types Ia, Ib, Ic, II, and III GBS by using a binding immunoassay in which MCA bound to bacteria was detected with a goat anti-mouse immunoglobulin conjugated to peroxidase. Four MCAs were selected with a high affinity in the enzyme immunoassay for one of the GBS serotypes, anti-GBS Ia/Ic, Ib, II, and III. The isotype of each of the MCAs was μk.

Preparation of ascites. BALB/c mice were pretreated with an intraperitoneal injection of pristane (2, 6, 10, 14-tetramethylpentadecane, Sigma Chemical Co., St. Louis, Mo.). One to four days later, 2×10^7 cells from each hybridoma cell line were suspended in 2 ml of serum-free media: Dulbecco modified Eagle medium, 10% NCTC (Microbiological Associates, Bethesda, Md.), 0.2 U of bovine insulin per ml, 0.45 mM pyruvate, and 1 mM oxaloacetate; and injected intraperitoneally. Paracentesis was performed every other day after 7 to 10 days. Mice were reinjected intraperitoneally with 2 ml of sterile media after each tap to prevent fluid shifts. The ascites was centrifuged at 2,000 rpm (800 × *g*) and stored at 4°C with 0.01% sodium azide.

Conjugation of peroxidase to antibody. Each MCA was conjugated to type VI horseradish peroxidase (Sigma). Briefly, peroxidase was activated by the addition of 0.2 ml of a 1% glutaraldehyde solution (Sigma) in 0.1 M phosphate buffer (pH 6.8). The solution was left at room temperature overnight and then dialyzed the following day at 4°C against 0.15 M NaCl to remove excess glutaraldehyde. Then 1 ml (5 mg) of MCA, purified by precipitation with 50% NH_4SO_4 solution (9) and dialyzed against 0.15 M NaCl to remove azide, was added to the peroxidase solution with 0.2 ml of 0.5 M sodium carbonate-bicarbonate buffer (pH 9.5), and the entire mixture was incubated at 4°C for 24 h. The next day, 0.1 ml of 1 M L-lysine (pH 7.0; Sigma) was added and the solution was dialyzed against phosphate-buffered saline (PBS) and 50% glycerol (Sigma). The total volume was brought to 2 ml with 50% PBS-glycerol and stored at 4°C.

Sandwich enzyme immunoassay: preparation of microtiter plates. A 50-μl amount of each MCA in unpurified ascites diluted 1:100 in 0.04 M borate buffer (pH 8.6) was added to polystyrene microtiter wells (Immulon I Removawell Strips, Dynatech Laboratories, Inc., Alexandria, Va.) and incubated overnight at 4°C. To prevent nonspecific binding, the plates were incubated the following morning for 4 h at room

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TABLE 1. Sandwich enzyme assay sensitivity: evaluation of the enzyme assay for detection of whole group B streptococci

Serotype	CFU/ml[a]	P[b]
Ia	5×10^5	10^{-5}
Ib	10^5	0.05
Ic	10^5	0.001
II	5×10^4	10^{-5}
III	5×10^4	10^{-4}

[a] Minimal bacterial concentration detected; assayed in 0.5 log dilution.

[b] The *P* value represents the optical density of the test bacterium in the sandwich enzyme assay versus that in a control well.

temperature with PBS (pH 7.2)–100 mM glycine–1% bovine serum albumin and stored at −20°C until needed.

Antigen preparation for assays. Reference strains of types Ia, Ib, Ic, II, and III GBS were inoculated into 10 ml of Todd-Hewitt broth and incubated overnight at 37°C. The following morning, the number of CFU per milliliter was quantitated by serial dilution of bacteria. The stock culture was placed at 4°C to prevent further growth. Bacteria were diluted from 10^4 to 10^7 in 0.5 log dilutions for the assay. To determine the sensitivity of the sandwich assay, type III purified native polysaccharide antigen (supplied by Dennis Kasper) was dissolved in PBS (pH 7.2) at dilutions from 0.1 ng/ml to 1 μg/ml in 1.0 log serial dilutions and then tested in the assay by using type III GBS MCA. Cerebrospinal fluid specimens from five patients with known GBS meningitis were also tested.

Assay. Bacteria (Ia, Ib, Ic, II, and III) or purified polysaccharide antigen (100 μl) diluted as described above was added to microtiter wells containing bound MCA with one of the following specificities (anti Ia/Ic, anti Ib, anti II, anti III) and incubated for 1 h at room temperature. Unbound antigen was removed by washing with PBS three times and then flicking the remaining fluid into the sink. The "sandwich" was completed by the addition of 100 μl of peroxidase-labeled type-specific antibody diluted 1:100 to 1:250 in PBS (99.9%)–Tween (0.05%)–gelatin (0.05%).

After a 1-h incubation, the wells were washed 10 times with PBS to remove unbound enzyme conjugate. Then 100 μl of substrate (10 μg of ortho-phenylene diamine; 10 ml of citrate buffer [pH 4.5; Sigma]; 20 μl of 3% hydrogen peroxide) was added to each well and incubated at room temperature in the dark for 20 min. The reaction was stopped by the addition of 25 μl of 0.1 M NaF to each well. Negative controls (Todd-Hewitt broth) were assayed, and unreacted substrate was used as a zero reference. The optical density of each well was determined on a multichannel spectrophotometer (MR 580 Microelisa AutoReader; Dynatech) at 450 nm. A specimen was considered positive (i.e., containing GBS antigen) if the optical density of the test well was significantly greater ($P < 0.05$) than that of a negative control. Each GBS serotype was tested against all four MCAs, and specimens were run in quadruplicate.

Statistical analysis. Optical densities of each test well were compared with negative controls by means of an independent *t* test.

RESULTS

The minimal numbers of CFU of each GBS serotype per milliliter detected with the enzyme assay are shown in Table 1. The sandwich enzyme assays for types II and III GBS were most sensitive and detected organisms at 5×10^4 CFU/ml. The assays for types Ia and Ib detected one log less bacteria. No cross-reactions were observed when each of the GBS bacterial serotypes was reacted with antibodies of the differing serotype specificities (except for Ia and Ic, which share a polysaccharide antigen) (Table 2). Type III purified native polysaccharide antigen diluted in PBS was detected at a concentration of 1 ng/ml (antigen optical density, 0.108 versus control optical density 0.034; $P < 0.05$). Cerebrospinal fluid specimens from five patients with known GBS III meningitis were positive in the enzyme assay (Table 3).

DISCUSSION

Early-onset GBS infection in neonates is a well-recognized clinical problem. Attempts to interrupt or prevent vertical transmission of GBS are complicated by the need for rapid identification of those mothers whose infants may develop infection. Although there are theoretical risks associated with the intrapartum administration of ampicillin to such a large group of women, there is a greater potential benefit of antibiotic administration, namely, decreased infant colonization with GBS (1, 7). Many immunodiagnostic assays have been developed to detect GBS antigen, including counterimmunoelectrophoresis, competitive enzyme assay (12), latex particle agglutination, immunofluorescence, and staphylococcal coagglutination. Most of these assays require antisera obtained from animals and detect group rather than type-specific determinants.

We have shown that the MCA enzyme immunoassay detects 5×10^4 to 5×10^5 CFU of bacteria per ml and 1 ng of type III GBS antigen per ml. In contrast, counterimmunoelectrophoresis and latex particle agglutination are less sensi-

TABLE 2. Cross-reactivities of GBS serotypes in sandwich enzyme assay

Bacterial serotype	Cross-reactivity in enzyme assays with the following serotype:							
	Ia/c		Ib		II		III	
	Optical density	P[a]	Optical density	P	Optical density	P	Optical density	P
Ia	1.5 ± 0.0007	$<10^{-3}$	0.0005 ± 0.0005	NS	0.033 ± 0	NS	0.029 ± 0.004	NS
Ib	0.059 ± 0.005	NS	1.5 ± 0	$<10^{-5}$	0.020 ± 0.009	NS	0.006 ± 0.002	NS
Ic	0.452 ± 0.036	<0.01	0.016 ± 0.022	NS	0.017 ± 0.005	NS	0.020 ± 0.001	NS
II	0.064 ± 0.003	NS	0.070 ± 0.026	NS	1.010 ± 0.0007	$<10^{-5}$	0.015 ± 0.001	NS
III	0.043 ± 0.008	NS	0.012 ± 0.002	NS	0.034 ± 0.004	NS	0.516 ± 0.005	$<10^{-4}$
THB negative control	0.051 ± 0.024		0.009 ± 0.004		0.031 ± 0.002		0.031 ± 0.002	

[a] *P* represents the optical density of the test bacterium in the sandwich enzyme assay versus the value in the control well. NS, Not statistically significant.

TABLE 3. Sandwich enzyme assay for type III GBS antigen in patients with known GBS meningitis

CSF[a] specimen no.	Native antigen concn (μg/ml)	OD[b]
1	0.8	0.687
2	6.8	0.966
3	1.7	1.004
4	6.8	0.997
5	0.8	0.540

[a] CSF, Cerebrospinal fluid.
[b] OD, Optical density.

tive and have minimum detection levels of 0.5 and 0.063 μg of type III antigen per ml, respectively. Quantitative data for the number of CFU per milliliter that each of these assays will detect are not available (2, 3, 6, 8). Staphylococcal coagglutination requires at least 6×10^8 CFU/ml; however, treatment of clinical specimens with nitrous acid greatly increases the sensitivity of the assay. Slifkin et al., using a micro-nitrous acid extraction method, detected GBS directly from 92% of women known to harbor this organism (16). Immunofluorescence can detect 10^5 CFU/ml; however, this is a tedious method and requires the use of a fluorescent microscope (15). In addition, immunofluorescence has not proved reliable for detecting GBS in clinical specimens containing mixed flora (4, 5). The sandwich enzyme assay is preferable to our previously described monoclonal antibody inhibition assay because of its positive endpoint (color production versus color inhibition) as well as a 10-fold increase in sensitivity. The MCA used in the enzyme immunoassay reacts with the single polysaccharide determinant unique to each serotype and has greater potential for epidemiological investigations. Although MCA could be substituted in any of these assay systems, enzyme assays offer greater sensitivity because they magnify the antigen-antibody reaction by catalyzing a large number of molecules of substrate. The reaction is visible to the naked eye and can be quantitated colorimetrically by using a spectrophotometer. MCA is more advantageous than heteroantisera because of its homogeneity, improved specificity, and availability from cell culture over long periods of time. The MCA enzyme immunoassay should prove useful for detecting colonization, serotyping bacterial isolates recovered by diagnostic microbiology laboratories, and identifying GBS antigen in body fluid specimens. The presence of soluble antigen in addition to whole bacteria in clinical infections should further increase the sensitivity of this assay system.

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