Radioimmunoassay for Measuring Antibodies Specific for Group B Streptococcal Types Ia, Ib, Ic, II, and III

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The object of this study was to develop a test that would measure antibodies directed against group B streptococcal types Ia, Ib, Ic, II, and III. The type-specific carbohydrate antigens were purified, labeled with ^{125}I , and used to develop a radioimmunoassay. This procedure should be particularly useful in testing human sera for group B type-specific antibodies, since it requires very small quantities of antigens and measures primary type antigen-antibody interactions.

No primary binding antigen-antibody test has been developed thus far to measure group B streptococcal type-specific antibodies. These antibodies are protective in mice (11) and therefore may be important in providing human immunity to group B infections. That immunity to group B streptococci is acquired by individuals is implied by the fact that disseminative group B disease rarely occurs in persons over age 2 (18) unless the host is compromised (12). Whether neonatal septicemia and meningitis may be—and usually are—prevented by passive immunization of the fetus is a timely question for the following reasons. Chemotherapy often comes too late for the infant with “early-onset” disease (7, 10); antibiotic prophylaxis of colonized mothers is of unknown efficacy (5); and antibiotic prophylaxis of colonized infants either does not always eradicate the streptococci or does not always prevent recolonization (15).

An association of certain group B types with disease syndromes has been noticed (1-3, 7, 18). Type III is isolated most frequently from cases of neonatal meningitis, regardless of the age of the infant at onset of the disease, and from cases of late-onset septicemia. There are conflicting reports, however, on which types are associated with early-onset septicemia (1, 7). These discrepancies could have been due to small sample sizes, local population differences, different culture techniques, or a combination of these factors. Our analysis of a large sample size with wide geographic and time boundaries supported Baker and Barrett's conclusion that all five types are associated with early-onset septicemia (1; H. W. Wilkinson, unpublished data).

The purpose of this study was to develop a sensitive antibody test for the group B disease-

producing types. The radioimmunoassay (RIA) technique was chosen because it allows one to make measurements that are extremely sensitive and specific. Antigens from all five group B types were included in the test because of (i) the association of all types with early-onset neonatal septicemia, a syndrome with a poor prognosis (3, 7, 10, 14), (ii) the isolation of all types from cases of late-onset septicemia and from cases of infant meningitis (albeit type III predominates), and (iii) the existence of common antigenic determinants among several group B types (10, 16). The latter fact makes a type-specific test unreliable unless it is carefully controlled with heterologous type antigens. Evidence is presented herein that the RIA can be used to measure group B streptococcal antibodies.

MATERIALS AND METHODS

Typing group B streptococci. Procedures for performing and interpreting the capillary precipitin test for determining group B types were described previously (18).

Purification of antigens. The five group B strains used as sources of the type-specific carbohydrate (CHO) antigens were: type Ia (strain 090), type Ib (H36B), type Ic (A909), type II (18RS21), and type III (D136C) (11, 17). Each antigen was extracted and purified as follows. Packed whole cells weighing 11 to 17 g were suspended in 130 ml of cold 2.5% trichloroacetic acid and subjected to 8 min of sonification (Bronwill Biosonik IV, 80% of maximum setting, large probe) in an ice bath. Centrifugation at 4°C was used to separate cells and debris from the supernatant fluid, which contained the type CHO antigen. The cells were washed four times with 10 ml of cold 2.5% trichloroacetic acid to collect any residual type CHO not obtained in the first centrifugation step. After centrifugation, all supernatant fluids with detectable type CHO were combined. The antigen was precipitated from the combined superna-

tant fluid by adding 2 volumes of cold 95% ethanol. The precipitate was collected by centrifugation and then resuspended in 20 ml of saline. Insoluble material was removed by centrifugation. The extract was neutralized with NaOH and tested for the presence of antigens by capillary precipitin and Ouchterlony analyses (16, 18). No group B antigen was detected, but, since the precipitin test is relatively insensitive, each type antigen was subjected to two additional 2-volume ethanol precipitation cycles to remove any remaining group B CHO (17). The absence of group B CHO was verified chemically by the absence of rhamnose in the preparation (4). The purified type CHO was suspended in distilled water and lyophilized. Yields, expressed as milligrams of CHO obtained per gram of cell mass, were: Ia CHO (type Ia), 1.6; Ib CHO, 1.5; Ic CHO (type Ic), 0.48; II CHO, 0.97; and III CHO, 0.5.

Labeling of purified antigens with ^{125}I . The method devised by Gotschlich et al. for meningococcal polysaccharides (9) was used to radiolabel each type-specific CHO antigen with ^{125}I (New England Nuclear Corp.). This method consisted of tyrosylation of the cyanogen bromide-activated CHO, followed by chloramine-T iodination. Each CHO antigen, in a concentration of 5 mg per ml of distilled water, was adjusted to pH 11.0 with 0.1 N NaOH and then was allowed to react with 0.5 ml of cyanogen bromide reagent (20 mg per ml of 0.1 N NaOH) for 5 min at room temperature. During this time, the pH was maintained at 11.0 by adding 0.1 N NaOH dropwise as needed. One milliliter of tyramine reagent (50 mg per ml of 0.5 N sodium bicarbonate) was added to the reaction mixture, which was then adjusted to pH 8.5 and allowed to sit overnight at room temperature. The tyrosylated antigen was dialyzed against distilled water for 24 h and then against 0.5 M phosphate buffer, pH 7.6, for 3 h. It was then diluted in the same buffer to a concentration of 1 mg of CHO per ml and was stored at -70°C . Two hundred micrograms of the thawed antigen, 0.5 mCi of ^{125}I , and 10 μl of chloramine-T reagent (20 mg per ml of 0.05 M phosphate-buffered saline [PBS], pH 7.3) were allowed to react for 10 min at room temperature, and then 50 μl of sodium hydrosulfite reagent (10 mg per ml of PBS) was added to stop the reaction. Ten microliters of potassium iodide reagent (200 mg per ml of PBS) was added, and the labeled antigen was rinsed into dialysis tubing with 300 μl of a 1:10 dilution of the iodide reagent. The antigen was dialyzed against PBS at 4°C until the radioactivity in the dialysate decreased to a level that stayed the same for 2 successive days. Then it was stored at 4°C until needed. For testing in the RIA, it was diluted in PBS containing 0.1% (wt/vol) bovine serum albumin to concentrations of 0.3, 3, and 30 $\mu\text{g}/\text{ml}$. Specific activities of the five type antigens ranged from 2,000 to 13,000 counts/min per ng of CHO.

Antisera. All sera were heated for 30 min at 56°C . The Biological Products Division, Center for Disease Control, provided the rabbit antisera for streptococcal groups A, B, and D; group B types Ia, Ib, II, and III; and normal control serum. Methods used for production of these reagents have been described (17). Representative rabbit antisera for each group

B type were obtained from Rebecca C. Lancefield, Rockefeller University. Dilutions of antisera from 1:10 to 1:10,000 were made in fetal calf serum (FCS) (KAM Laboratories Inc., Grandview, Mo., or KC Biologicals Inc., Lenexa, Kan.).

RIA. The double-label technique for measurement of radioactive antigen binding, described by Gotschlich et al. for meningococcal polysaccharides (8, 9), was used in the group B type-specific RIA. This modification of the Farr technique (6, 13) includes a ^{22}Na volume marker in the reaction mixture to make possible the use of small volumes that need not be accurately measured and requires fewer manipulations. In the present study, 1 μCi of ^{22}Na was added per ml of antigen solution used in the test. Ten microliters of the antigen- ^{22}Na solution was added to 10 μl of diluted antiserum in a 400- μl Microfuge tube (Beckman Instruments, Inc.). The tube was centrifuged in a model 152 Microfuge (Beckman Instruments, Inc.) for 1 min, held briefly on a Vortex Jr. mixer (Scientific Industries, Inc.), and refrigerated overnight. Twenty microliters of 80% saturated ammonium sulfate, held at room temperature, was then added to each tube, and the contents of the tube were mixed and refrigerated for 30 min. After a final 5-min centrifugation, most of the supernatant fluid was discarded. Each vial was counted three times in a Packard gamma counter with one channel set to count ^{125}I and the other set to count ^{22}Na . The average of the three counts per minute was converted to percentage of antigen bound by the experimental serum (%Bo. exp.) with the formula $[(Ea-eA)/100]/[(a-e)A]$, where E is counts per minute of ^{125}I experimental, e is counts per minute of ^{22}Na experimental, A is counts per minute of ^{125}I in the 10 μl of antigen added (control tube), and a is counts per minute of ^{22}Na in the same control tube (8). Corrections were made for the nonspecific binding of each antigen to FCS by using the formula

% bound value corrected =

$$100 - \frac{(100 - \% \text{Bo. exp.})100}{100 - \% \text{Bo. FCS}}$$

RESULTS

Development of type-specific RIAs. Figure 1 shows representative antigen-binding curves obtained when type-specific immune rabbit sera were reacted with the homologous antigens. High percent bound values were obtained with each antigen at concentrations of both 3 and 30 $\mu\text{g}/\text{ml}$. The antigen concentrations and antiserum dilution factors chosen for the test, however, were those that gave maximal differences in binding of homologous and heterologous antisera. Figure 2 shows the results of testing each antigen with homologous type and heterologous type, group, and normal sera. (Concentrations are given in the legend.) Heterologous binding was always at least 39% lower than was homologous binding. It appeared from these data that a type-specific RIA was possible.

FIG. 1. Antigen-binding capacity of group B streptococcal type-specific antisera. The group B type strain used to produce the antiserum was also used as the antigen source for the RIA. Antigen concentrations were: 3 μ g of Ia CHO per ml and 30 μ g of II or III CHO per ml.

FIG. 2. Difference in percentage of type CHO bound by homologous type-specific sera and heterologous type, groups B, A, and D, and normal sera. The five radiolabeled type antigens are indicated at the top of the figure. The eight sera listed below the Ib graph are depicted in the same order in the other four graphs: types Ia, Ib, II, and III; groups B, A, and D; and normal serum. Closed circles represent the percentage of type CHO bound by homologous type sera. Open circles represent the percentage bound by heterologous sera. Concentrations of antigens were: 3 μ g of Ia or Ib CHO per ml and 30 μ g of II or III CHO per ml. Antisera that were tested with the Ia CHO were diluted 1:1,000; those tested with the Ib, II, or III CHO were diluted 1:100.

Sensitivity and specificity of the RIA. The antisera used in the preceding experiments were type specific by capillary precipitin tests. That is, they contained antibodies to the major

type CHO antigen but no detectable antibodies to the antigenic determinants known to be commonly shared by several group B types (11, 16; Table 1): the Iabc CHO determinant of types Ia, Ib, and Ic; the Ibc proteins of types Ib and Ic; and a determinant of unknown chemical composition shared by types II and III (Rebecca C. Lancefield and Carol J. Baker, personal communications). Unabsorbed antisera raised against any type I strain usually give positive precipitin reactions with extracts of all three of the type strains Ia, Ib, and Ic. Similarly, unabsorbed type III antisera sometimes react with extracts of type II strains. Table 1 provides a summary of the antigenic determinants that are presently known. Tables 2 through 5, described below, show results of the RIA with group B typing antisera obtained from Rebecca C. Lancefield. All but one (see Table 5, footnote a) of these antisera were unabsorbed and therefore should illustrate reactions due to shared determinants, in addition to those due to the major type-specific CHO antigens. It should be pointed out that the Ibc protein antigens (17) were removed during purification of the CHO

TABLE 1. Multiplicity of antigenic determinants in group B types

Group B type designation	Known type antigens ^a	Specificities found in homologous immune serum ^b
Ia	Ia CHO	Anti-Ia (specific) Anti-Iabc (common determinant)
Ib	Ib CHO Ibc proteins	Anti-Ib (specific) Anti-Iabc Anti-Ibc
Ic	Ia CHO Ibc proteins	Anti-Ia (specific) Anti-Iabc Anti-Ibc
II	II CHO ^c	Anti-II (specific)
III	III CHO ^c	Anti-III (specific) Anti-II-III (common determinant)

^a CHO antigens extracted by hot HCl are composed of galactose, glucosamine, and, occasionally, glucose. Those extracted by saline or trichloroacetic acid contain sialic acid, in addition to the hexoses and hexosamines (11, 16; for type III, Carol J. Baker, personal communication).

^b Sera from different rabbits differ with respect to diversity and strength of these antibodies. Antibodies directed toward the sialic acid portion of some of the antigens have also been found.

^c Occasional type II and rare type III strains also contain Ibc proteins. They have not been studied extensively.

antigens, and therefore Ibc antibodies should not participate in the RIA.

Table 2 shows the results of testing type I antisera that had no Iabc antibodies detectable by precipitin tests. That is, each antiserum was specific for either Ia or Ib CHO but did not react in the precipitin test with both antigens. Two of the sera, however, contained nonprecipitating Iabc antibodies (no. 14 and 58) since these sera bound both antigens in the RIA. At suitable dilutions of type I sera, no cross-reactions occurred with the II or III CHO. Table 3 provides additional support for these observations by showing that type I antisera containing Iabc antibodies bound both Ia and Ib but not II and III CHO.

The next experiments were concerned with the specificities of the type II and III RIA. Table

TABLE 2. Percentage of radiolabeled CHO antigens bound by type I antisera containing specific precipitins^a

[¹²⁵ I]CHO antigen	Rabbit antiserum					
	Type Ic no. 55		Type Ib no. 14		Type Ib no. 58	
	10 ^{-2b}	10 ⁻³	10 ⁻²	10 ⁻³	10 ⁻²	10 ⁻³
Ia	65	68	69	38	44	29
(type Ia)						
Ia	86	69	50	37	40	12
(type Ic)						
Ib	24	0	68	56	53	33
II	44	11	0	2	0	0
III	0	0	0	0	0	0

^a Capillary precipitin reactions (strength of reaction based on scale of 1 + [weak] to 4 + [strong] [reference 17]) of undiluted antisera with HCl extracts of group B types Ia, Ib, Ic, II, and III: no. 55, 3 + Ia, 4 + Ic; no. 14, 4 + Ib; no. 58, 3 + Ib.

^b Dilution factor.

TABLE 3. Percentage of radiolabeled CHO antigens bound by type I antisera containing Iabc common determinant precipitins^a

[¹²⁵ I]CHO antigen	Rabbit antiserum					
	Type Ia W3179		Type Ib no. 51		Type Ic no. 39	
	10 ^{-2b}	10 ⁻³	10 ⁻²	10 ⁻³	10 ⁻²	10 ⁻³
Ia	77	73	85	84	100	87
(type Ia)						
Ia	75	54	66	66	75	32
(type Ic)						
Ib	64	66	100	100	98	100
II	0	2	13	10	28	22
III	10	0	0	0	0	0

^a Capillary precipitin reactions of undiluted antisera with HCl extracts of group B types Ia, Ib, Ic, II, and III: W3179, 3 + Ia, 2 + Ib, 3 + Ic; no. 51, 4 + Ia, 4 + Ib, 4 + Ic; no. 39, 4 + Ia, 4 + Ib, 4 + Ic.

^b Dilution factor.

4 shows results of a type II test that was highly specific since no CHO other than II was bound by type II antisera. Similarly, two type III antisera (Table 5) bound only the homologous III antigen. A third serum, R544, was used to illustrate the observations made by R. C. Lancefield and C. J. Baker (personal communications) that some type III antisera contain antibodies directed against antigenic determinants common to both type II and type III. Three absorptions of this serum with type II cells were required to remove the II-III common antibodies. Type III-specific antibodies remained in the absorbed serum.

The results described in Tables 2 through 5 and Fig. 1 and 2 are illustrative but representative examples of experiments done to develop a group B type-specific RIA. From the results on

TABLE 4. Percentage of radiolabeled CHO antigens bound by type II antisera^a

[¹²⁵ I]CHO antigen	Rabbit antiserum					
	E1		W4618		R2151	
	10 ^{-2b}	10 ⁻³	10 ⁻²	10 ⁻³	10 ⁻²	10 ⁻³
Ia	19	12	0	0	0	3
(type Ia)						
Ia	0	0	6	6	0	0
(type Ic)						
Ib	0	0	0	0	0	0
II	79	50	69	17	72	46
III	0	0	0	0	0	1

^a Capillary precipitin reactions of undiluted antisera with HCl extracts of group B types Ia, Ib, Ic, II, and III: 4 + II.

^b Dilution factor.

TABLE 5. Percentage of radiolabeled CHO antigens bound by type III antisera^a

[¹²⁵ I]CHO antigen	Rabbit antiserum							
	R2237		R544				W4599	
	10 ^{-2b}	10 ⁻³	No. 74		No. 68a		10 ⁻¹	10 ⁻²
Ia	14	0	3	9	0	0	12	0
(type Ia)								
Ia	0	4	0	0	9	0	24	0
(type Ic)								
Ib	0	0	0	0	0	0	0	0
II	2	9	60	26	37	19	17	9
III	54	51	48	25	54	63	55	47

^a Capillary precipitin reactions of undiluted antisera with HCl extracts of group B types Ia, Ib, Ic, II, and III: R2237, 4 + III, 1 + II; no. 74 (absorbed twice with type II cells), 2 + III, 2 + II; no. 68a (absorbed three times with type II cells), 3-4 + III; W4599, 1 + III.

^b Dilution factor.

169 duplicate specimens, the within-specimen standard deviation was estimated to be $s_w = 6.3\%$. A homologous mean value of 67% (range, 46 to 100%) and a heterologous mean value of 5% (range, 0 to 28%) were obtained with 46 and 47 paired specimens, respectively.

DISCUSSION

The purpose of this study was to develop an antibody test to measure antibody-mediated immunity, if it occurs, to group B streptococcal infections. The RIA technique was chosen for its sensitivity and its ability to detect primary type antigen-antibody reactions. Secondary type immunological reactions, such as those seen in precipitin or hemagglutination tests, often fail to detect total antibody (13).

The RIA was developed with the five type CHO antigens extracted with cold trichloroacetic acid from sonified streptococcal cells. Although larger antigen yields are obtained with hot HCl extraction, this method yields antigens of lower molecular weight and hydrolyzes labile sialic acid determinants that could be important in eliciting protective immunity (11, 16). The Ibc protein antigens (16, 17) were excluded from the study although they also are protective in mice (11). Future studies will be directed toward including them in the RIA.

A modification of the Farr technique by Gotschlich (8) to measure meningococcal antibodies was adapted for measuring group B type antibodies. The purified CHO antigens were labeled with ^{125}I and tested in concentrations of 0.3 to 30 $\mu\text{g}/\text{ml}$ with rabbit antisera specific for group B types Ia, Ib, Ic, II, and III; groups B, A, and D; and normal control serum. Differences of at least 38% were obtained in the antigen-binding capacity of homologous and heterologous sera at their optimal dilutions of 1:100 or 1:1,000. The test detected antibodies directed against the major type-specific CHO determinants and those directed against antigenic determinants commonly shared by several types; both kinds of antibodies are protective in mice (11). The test also detected non-precipitating antibodies in addition to those detected by the capillary precipitin test.

The RIA may provide a tool for getting answers to several questions regarding group B disease in humans. First, do human sera contain type-specific antibodies? Are they protective and, if so, at what concentrations? Second, if protective antibodies occur, can they cross the placenta and thus passively protect the neonate? And third, does cross-protection among several types occur in humans as it does in mice? The answers to these questions will be

obtained only after testing large numbers of sera from both ill and well individuals.

The interpretation of RIA reactions with human sera must be made cautiously because of the finding in the present study that unabsorbed rabbit antisera often bind the type antigens of several group B types that contain common antigenic determinants. For example, it would not be surprising if an antigenic stimulus by any one of the three type I strains induced an antibody response to types Ia, Ib, and Ic, nor would it be surprising if types II and III elicited cross-reactive antibodies. Because of the potential complications in interpreting the binding of any one antigen by sera of unknown specificity, studies of human sera will be made with the purified antigens of all five group B types.

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