Identification of Group B Streptococci by Immunofluorescence Staining

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Gamma globulin fractions of rabbit antisera prepared with whole cell vaccines of group B types Ia, Ib, II, and III and labeled with fluorescein isothiocyanate stained group B streptococci type specifically. Type Ic cells, which contain the Ia polysaccharide antigen of type Ia and the Ic protein antigen of type Ib, were specifically stained by both Ia and Ib conjugates. A group B conjugate pool (B pool) that contained one conjugate specific for each group B type at its predetermined titer gave positive fluorescent-antibody (FA) reactions (4+ intensity) with group B stock strains and negative FA reactions (less than 2+ intensity) with stock strains of streptococcal groups A, C through H, and K through U, viridans streptococci, Streptococcus pneumoniae, Staphylococcus aureus, Neisseria gonorrhoeae, and representative Enterobacteriaceae. Examination of 883 clinical isolates submitted to the Streptococcus Laboratory (Center for Disease Control, Atlanta, Ga.) for identification revealed a 99.1% agreement between FA and culture-precipitin methods. All 305 group B streptococci identified by culture-precipitin and six nonhemolytic group B streptococci missed initially by culture tests were identified correctly by FA. Results of cultural and FA methods in a double-blind study of 99 vaginal swabs agreed on 96 of 99 strains. Three nonhemolytic group B streptococci were identified first by FA and later confirmed by culture-precipitin tests.

Group B streptococci have long been recognized as significant pathogens in bovine mastitis and as opportunistic invaders of certain compromised human hosts (5, 16). More recently, their role in neonatal disease has been emphasized, particularly in neonatal meningitis, a disease that has two syndromes: the rapidly fulminating with sudden onset of symptoms and a high mortality rate and the more insidious with delayed onset (> 10 days) and a better prognosis (1-3, 6, 7, 10). Death often precedes diagnosis of the acute form of the disease and identification of the Streptococcus (1, 10). These infants usually obtain the organism from their mothers' vaginas during birth or, less frequently, by nosocomial transmission (1, 10). Epidemiological factors concerning late onset group B meningitis are unknown. Further, reported carrier rates in gravid females (2, 10) vary widely, perhaps because of demographic differences, but more likely because of culture frequency and laboratory procedures. The attack rate in infants seems low (1, 10), and this has prompted some clinicians to advise against the indiscriminate use of antibiotic prophylaxis (2, 8).

The prognosis of neonatal meningitis is improved by prompt and adequate treatment (19), which in turn depends on rapid identification of the streptococci. Conventional methods used to identify group B streptococci require a minimum of 2 days for presumptive tests (9) or 3 days for serological grouping and typing. The fluorescent-antibody (FA) technique seemed particularly suitable for identifying group B streptococci in a clinical situation because of its rapidity and sensitivity, because many laboratories are already equipped for its use, and because its reliability has been established by its widespread use in the identification of group A streptococci.

MATERIALS AND METHODS

Preparation of antisera. Preliminary data suggested that group B cells are stained by a group B conjugate only if the fluorescein isothiocyanate (FITC)-labeled antibodies are specific for the type antigens present on the surface of the group B cells. Four of the five group B types were used in vaccines: type Ia (strain 090), type Ib (H56B), type II (18RS21), and type III (D136C). Type Ic vaccine was not used, since both of the type antigens in this strain are present in two other types: Ia polysaccharide antigen...
in type Ia and Ic protein antigen in type Ib (25, 27). Vaccines were prepared by Lancefield's method (15), modified by first digesting type III cells with 0.06% pepsin (18) to eliminate the R protein antigen of this strain (24). This antigen occurs among streptococci of various groups and types (17, 22, 24) that might stain non-specifically with conjugates that contain anti-R antibodies (13). Each of 20 rabbits was inoculated with only one vaccine. After several immunization series, antisera were tested by the capillary precipitin test (26) by using HCl extracts of homologous group B strains. Those that gave strong (4+) precipitin reactions were labeled individually with FITC.

**Preparation and conjugation of globulin.** Each antisera was treated separately. Gamma globulin was precipitated by 70% ammonium sulfate (12) and resuspended to the original serum volume. FITC, obtained from McKinnon (Center for Disease Control [CDC], Atlanta, Ga.), was allowed to react with each globulin fraction for 4 h at room temperature. Fluorescein-to-protein ratios were determined for each conjugate (12). An electrophoretic analysis was done to assure that most of the FITC was bound and that the preparations were essentially free of albumin which can compete for FITC and thus cause non-specific staining (11). Conjugates placed on cellulose acetate electrophoresis membranes were subjected to electrophoresis for 30 min in the Beckman Microzone apparatus. Bands were detected by staining with Pronase S and densitometry or by fluorescence of bound FITC under ultraviolet radiation (Woods lamp).

**Testing individual conjugates.** The four vaccine strains were grown at 35 C in 5 ml of Todd-Hewitt broth (Difco) for 18 h. Smears were made directly from the well-shaken broth cultures, allowed to air-dry, and fixed for 2 min with 95% ethanol. Serial dilutions of each conjugate were prepared in 0.01 M phosphate-buffered saline, pH 7.4. Cells representing homologous and heterologous types were allowed to react with one drop of each conjugate dilution for 15 to 30 min in a moist chamber at room temperature. The smears were washed and examined by established methods (20, 21). Unstained smears and those stained with FITC-labeled normal rabbit globulin served as controls. The titer of each conjugate was the reciprocal of the highest dilution that gave no lower than 3 to 4+ fluorescence of group B cells. Fluorescence intensity was recorded on a scale of from 1+ to 4+ (20).

**Preparation and testing of group B conjugate pool.** Ten conjugate pools were made. Each pool contained one conjugate each of types Ia, Ib, II, and III at a final dilution representing its predetermined titer. Titters were then determined for each of the pools prepared. Pools were tested with individual cells of each type and with a pool of the vaccine strains. Electrophoretic and fluorescein-to-protein analyses were performed on each pool. One pool was selected for additional studies.

**Evaluation of group B conjugates with stock strains.** The B conjugate pool (B pool) and its component type-specific conjugates were tested with stock strains of streptococcal groups A through U, viridans streptococci, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, and representative *Enterobacteriaceae*.

**Evaluation of group B conjugate pool (B pool).** Eight hundred and eighty-three consecutive isolates submitted for identification to the Streptococcus Laboratory, CDC, were tested with a 1:2 dilution of the B pool. FA results were compared with those obtained with conventional culture-precipitin methods in the Streptococcus Laboratory. The culture methods included determination of hemolysis by viewing deep colonies in 5% rabbit blood agar pour plates with a dissecting microscope and determination of serological group and type by the capillary-precipitin test using Lancefield extracts of beta-hemolytic strains (23, 26). Todd-Hewitt broth cultures of 99 vaginal swabs obtained in an unrelated study were tested similarly in a double-blind study. Cells that stained at a 3 to 4+ level of intensity were considered positive in all studies.

**Preservation and storage of conjugate.** Conjugates were preserved with merthiolate (1:10,000) and stored at 4 C. Stability of the B pool was tested weekly with vaccine strains and fresh isolates.

**RESULTS**

Twenty antisera (five sera for each of the four vaccine strains) were conjugated with FITC and titered (Table 1). Fluorescein-to-protein ratios ranged from 5 to 18 and titers from 2 to 32.

**Table 1. Characteristics of FITC-labeled group B streptococcal antisera**

| No. | Vaccine strain | Type designation | Mg of protein per ml of serum | µg of bound FITC per ml of serum | Titer |
|-----|----------------|------------------|-----------------------------|---------------------------------|-------|
| 1   | 90             | Ia               | 9.0                         | 125                             | 13.8  | 16    |
| 2   | 90             | Ib               | 8.0                         | 105                             | 13.2  | 16    |
| 3   | 90             | Ia               | 11.5                        | 140                             | 12.1  | 16    |
| 4   | 90             | Ib               | 7.5                         | 90                              | 12.0  | 4     |
| 5   | 90             | Ia               | 11.3                        | 130                             | 11.3  | 8     |
| 6   | 90             | Ib               | 12.0                        | 140                             | 11.6  | 16    |
| 7   | 90             | Ib               | 7.5                         | 80                              | 16.0  | 16    |
| 8   | H36B           | Ib               | 5.0                         | 90                              | 18.0  | 16    |
| 9   | 90             | Ib               | 12.5                        | 145                             | 11.6  | 32    |
| 10  | 90             | Ib               | 10.5                        | 120                             | 11.4  | 32    |
| 11  | 18RS21         | II               | 32.0                        | 160                             | 5.0   | 16    |
| 12  | 18RS21         | II               | 11.0                        | 140                             | 12.7  | 32    |
| 13  | 18RS21         | II               | 5.0                         | 50                              | 10.0  | 16    |
| 14  | 18RS21         | II               | 5.0                         | 60                              | 12.0  | 8     |
| 15  | 18RS21         | II               | 7.5                         | 50                              | 8.3   | 8     |
| 16  | D136C          | III              | 10.0                        | 105                             | 10.5  | 2     |
| 17  | D136C          | III              | 7.0                         | 100                             | 14.2  | 2     |
| 18  | D136C          | III              | 10.5                        | 110                             | 10.4  | 2     |
| 19  | D136C          | III              | 11.5                        | 110                             | 9.6   | 2     |
| 20  | D136C          | III              | 11.0                        | 145                             | 13.1  | 4     |

*µg of bound FITC/ml/mg of protein/ml.

*Expressed as the reciprocal of the highest dilution of serum that stained strains of homologous type >3+.
Table 2 shows that, although no absorptions of conjugates were done, each conjugate was type specific. It seemed likely that a pool of type-specific conjugates mixed in equal proportions would not stain all types with equal fluorescence intensities. Therefore, pools were prepared that would contain each individual conjugate at its own titer. Several pools stained all group B vaccine strains 3 to 4+ or greater when the pools were diluted 1:4. The pool selected for further study contained type Ia serum no. 2 (1:16 final dilution), type Ib serum no. 9 (1:32), type II serum no. 12 (1:32), and type III serum no. 20 (1:4). The pool was diluted 1:2 before use. Densitometric traces and ultraviolet scans of type-specific and pooled conjugates subjected to electrophoresis revealed that in each conjugate most of the protein was globulin and that most of the FITC was bound to the globulin fraction (Fig. 1).

Stock strains representing all Lancefield groups, viridans streptococci, pneumococci, and several other genera of bacteria frequently isolated from the genitourinary tract were used to test the specificity of the B pool (Table 3). All group B streptococci stained with 4+ intensity. All other organisms tested gave FA reactions of 2+ or less. Therefore, the same visual criteria could be used for a positive group B as for a positive group A FA reaction (20). Figure 2 shows the fluorescence of a typical group B *Streptococcus*.

The group B FA reagent was tested further by staining smears of 883 cultures isolated from clinical material and submitted to our laboratory for identification and by comparing the results with those obtained by conventional culture-precipitin techniques (Table 4). All 305 group B streptococci stained with an intensity of 3 to 4+. All other organisms gave negative reactions (2+ or less) except for five of 340 group A and three of 41 group C streptococci. Cross-reactions of group A conjugate with streptococcal groups C and G and *S. aureus* can usually be eliminated by absorption with group C cells or by a one-step inhibition either with group C antiserum or with pools of normal rabbit serum (20, 21). Absorption of the B pool with group C cells, however, diminished the group B reaction to an unacceptable level. Similarly, diluting the pool enough to reduce the cross-reaction also

### Table 2. FA reactions of individual conjugates with group B vaccine strains

| Conjugate no.* | Fluorescence observed with cells* of type: |
|----------------|------------------------------------------|
|                | Ia | lb | Ic | II | III |          |
| 1              | 4+ | 1+ | 4+ | 1+ | #   |          |
| 2              | 4+ | 1+ | 4+ | #  | #   |          |
| 3              | 4+ | 1+ | 4+ | #  | #   |          |
| 4              | 4+ | #  | 4+ | #  | #   |          |
| 5              | 4+ | #  | 4+ | #  | #   |          |
| 6              | #  | 4+ | 4+ | #  | 1+  |          |
| 7              | #  | 4+ | 4+ | #  | #   |          |
| 8              | #  | 4+ | 4+ | #  | #   |          |
| 9              | #  | 4+ | 4+ | #  | #   |          |
| 10             | #  | 4+ | 4+ | #  | #   |          |
| 11             | #  | #  | 4+ | #  | #   |          |
| 12             | #  | #  | 4+ | #  | #   |          |
| 13             | #  | #  | 4+ | #  | #   |          |
| 14             | #  | #  | 4+ | #  | #   |          |
| 15             | #  | #  | 4+ | #  | #   |          |
| 16             | #  | #  | #  | 4+ | #   |          |
| 17             | #  | #  | #  | 4+ | #   |          |
| 18             | #  | #  | #  | 4+ | #   |          |
| 19             | #  | #  | #  | #  | 4+  |          |
| 20             | #  | #  | #  | #  | 4+  |          |

*Each conjugate diluted to titer shown in Table 1, last column.

*Vaccine strains: type Ia (090), type Ib (H36B), type Ic (A909), type II (18RS21), type III (D136C).

### Table 3. FA reactions of group B conjugate pool with homologous and heterologous stock strains

| Stock strains tested                  | Fluorescence observed with B pool diluted 1:2 |
|---------------------------------------|---------------------------------------------|
| *Streptococcus* group A               | <2+                                         |
| group B                               | 4+                                         |
| group C                               | <2+                                         |
| group D                               | <1+                                         |
| group G                               | <1+                                         |
| groups E, F, H, and K through U       | <1+                                         |
| *Streptococcus pneumoniae*            | <1+                                         |
| *Staphylococcus aureus*               | <2+                                         |
| *Neisseria gonorrhoeae*               | <1+                                         |
| *Enterobacteriaceae*                  | <1+                                         |
reduced the homologous reaction. Adding either normal rabbit serum or group C antiserum blocked the reaction with the five group A strains but not with the three cross-reacting group C strains. However, they represented only 7% of the group C strains tested and 0.3% of all the strains tested. There was complete agreement between results of FA and culture-precipitin tests with all other isolates. Two observations deserve mention. First, culture-precipitin methods failed to identify the group B streptococci in several mixed cultures in which the relative numbers of B streptococci were small. Positive FA reactions were confirmed by isolation of single colonies, followed by growth and HCl extraction of the streptococci. Second, culture-precipitin methods did not identify several nonhemolytic group B streptococci. Positive FA reactions were later confirmed by precipitin and biochemical tests.

Todd-Hewitt broth cultures of vaginal swabs that had been collected in a study by the Bureau of Epidemiology, CDC, were used to test further the specificity of the B conjugate. Table 5 shows the results of this double-blind study; the results of FA and cultural methods agreed on 96 of 99 strains. No strains positive by culture were missed by the FA technique. The three strains positive by FA but missed initially by culture procedures were nonhemolytic group B streptococci that were later confirmed in the precipitin test.

Weekly stability tests showed that the group B conjugate pool was stable at 4°C for at least 6 months.

DISCUSSION

These data suggest that identification of group B streptococci by the FA technique is feasible. Data obtained in a clinical setting are needed to show if the technique is efficacious. Data thus far on prevalence, incidence (1, 2, 16), prophylaxis, treatment (2, 6, 8), and immunity (6, 16) indicate that the technique would be useful.

Five precautions must be taken to produce a sensitive specific group B conjugate. First, the conjugate must contain labeled antibodies specific for antigens on the surface of the streptococcal cell, i.e., the type antigens. Moody and Wilkinson (unpublished data) found that conjugated group B antisera often failed to give a positive FA reaction unless type-specific antibodies were included in the conjugate. Each antiserum should be labeled and titered individually, and its final dilution in the pool should be its predetermined titer; this would assure the same fluorescence intensity for each
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Table 4. Numbers of strains of gram-positive bacteria isolated from clinical material and listed by fluorescence intensities with group B conjugate pool

| Identity of bacteria* | No. of isolations with fluorescence intensities of: | Total no. of strains |
|-----------------------|---------------------------------------------------|----------------------|
|                       | None 1 to 2+ 3 to 4+                               |                      |
| Streptococcus group B type |                                                   |                      |
| Ia                    | 47 18 8                                        | 340                  |
| Ib                    | 57 36 120                                      |                      |
| Ic                    | 57 36 120                                      |                      |
| II                    | 57 36 120                                      |                      |
| II/IC                 | 57 36 120                                      |                      |
| III                   | 120 120                                        |                      |
| Nontypable            | 8 8 8                                          |                      |
| Ia and Ib mixed       | 11 11                                           |                      |
| Streptococcus group A |                                                   |                      |
| A                     | 166 169 5 3 41                                  | 340                  |
| C                     | 18 20                                           | 41                   |
| D                     | 40 1                                            | 41                   |
| F                     | 12 1                                            | 13                   |
| G                     | 12 6                                            | 18                   |
| K                     | 2 1                                             | 3                    |
| Alpha-hemolytic streptococci | 65 1 6* 15                                     |                      |
| Nonhemolytic streptococci | 9 6* 15                                         |                      |
| Streptococcus pneumonia | 17 17                                           |                      |
| Staphylococcus aureus  | 6 18                                            | 24                   |
| Total                 | 883                                             |                      |

* By culture and precipitin methods.
* These were eventually identified as group B streptococci by culture-precipitin tests.

Type and a comparable reduction in nonspecific staining and cross-reactions by dilution. Second, the conjugate cannot contain antibodies specific for R protein antigens that occur in other groups. Only a 63% agreement between results of FA and culture techniques was obtained in a study with anti-R-containing conjugates (14). Third, the conjugate should contain antibodies specific for the group B antigen, since occasional nontypable B strains are found. They represent approximately 1% of the group B streptococci isolated from human sources (unpublished data). The B pool used in this study contained group B antibodies present in the four constituent conjugates and thus stained nontypable strains. Strains that contained type antigens, however, failed to stain with conjugates other than those made with homologous type antisera. These observations support the hypothesis that type antigens on the surface of the streptococcal cell block the group B antigen-antibody reaction, perhaps by steric hindrance. Fourth, conjugates should be free of albumin and unbound FITC to reduce nonspecific staining and fluorescence. Fifth, and last, group B conjugates must be tested with large numbers of heterologous organisms to assure specificity.

In the present study, no cross-reactions occurred with the stock strains tested. Only eight cross-reactions occurred in testing 982 clinical specimens: five with group A streptococci which were blocked with either group C antiserum or pooled normal rabbit serum and three with group C streptococci which could not be blocked, for unknown reasons. These "false-positive" reactions were offset by the detection, with FA, of nine nonhemolytic group B streptococci which were initially not detected by culture techniques. These organisms occasionally occur in clinical material (28). Their identification by the CAMP test (4) may be less accurate than by FA, since results from another study (unpublished data) indicate that one of four nonhemolytic group B streptococci tested did not enhance the hemolysis produced by staphylococcal beta hemolysin on sheep blood agar plates.

A method in which a carefully prepared and tested group B FA reagent is used has one important advantage over conventional culture-precipitin methods. It requires much less time. A significant reduction in the time required to identify group B streptococci (several hours rather than several days) could mean more effective therapy for infants with neonatal meningitis. In addition, properly prepared type conjugates should allow typing of group B streptococci for epidemiological purposes.

The availability of these reagents commercially would determine the ultimate usefulness of group B immunofluorescence. Both group A conjugates and group B antisera vary in quality from commercial sources. Many "group B"

Table 5. Numbers of organisms identified as group B streptococci by FA and culture-precipitin tests in a double-blind study

| Culture precipitin | FA | Total |
|-------------------|----|-------|
|                   | +  | -     |      |
| +                 | 27 | 0     | 27   |
| -                 | 3  | 69    | 72   |
| Total             | 30 | 69    | 99   |
antiserum are type specific. We found one commercially prepared group B conjugate during our study; it was unsatisfactory in our tests.

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