Clinical Application of Immunofluorescence

I. Grouping β-Hemolytic Streptococci

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Received for publication 3 August 1964

ABSTRACT

SMITH, THOMAS B. (Armed Forces Institute of Pathology, Washington, D.C.). Clinical application of immunofluorescence. I. Grouping β-hemolytic streptococci. J. Bacteriol. 89:198-204. 1965.—Procedures are described for the production of antistreptococcal serum in rabbits and for the preparation of group-specific conjugates for Lancefield groups A, C, and G. A modification of the conventional technique of absorption and inhibition to prevent cross-reactions with common antigens was used with excellent results. In addition, a promising new approach to eliminating cross-reactions of group A conjugate with antigens of groups C and G by dilution with group A-variant antiserum was tested. A complete method is introduced that enables the clinical laboratory to report whether group A streptococci are present in a given throat culture well within 24 hr after the physician collects the sample.

Although the application of immunofluorescence to the identification of certain bacterial antigens has been a reliable procedure since the definitive work of Coons and Kaplan (1950), today it is still mainly a research tool. The failure of most of the larger clinical laboratories to utilize this new immunological approach, at least in the grouping of β-hemolytic streptococci, is even more enigmatic in view of the many papers in the field (Marshall, Eveland, and Smith, 1958; Moody, Ellis, and Updike, 1958; Redys, Ross, and Borman, 1960; Beutner, 1961; Peeples, Spielman, and Moody, 1961; Smith, Metzger, and Hoggan, 1962) published since 1958, the year Riggs et al. (1958) reported the synthesis of a new and easy-to-manage protein-labeling agent.

As already indicated, probably the simplest procedure to use as a beginning in the field is grouping β-hemolytic streptococci by immunofluorescence. Although this is an application that has been given rather wide discussion in the current literature, no investigator has reported approaching it from the viewpoint of the clinical laboratory. Considering this omission to be the probable reason for the dearth of immunofluorescence in the clinical laboratory, a project to study its effectiveness and practicality in grouping β-hemolytic streptococci isolated from throat cultures was instituted at the Armed Forces Institute of Pathology in February, 1963.

MATERIALS AND METHODS

Groups. Strains of the three groups of β-hemolytic streptococci that formed the basis of this study were secured from Roger M. Cole, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service. By use of strains 61X101 (A), 26RP66 (C), and 86X227 (G), immunizing antigens were prepared by inoculating 250 ml of Todd-Hewitt broth with each group and incubating at 37 C for 24 hr. The growth from each group, after centrifugation, was resuspended in 50 ml of sterile saline in a vaccine bottle and inactivated by heating in a water bath at 50 C for 45 min. Antigen concentration was found to be adequate when a sample, contained in a Corex rectangular cell, gave an optical-density reading of at least 1.0 with the Beckman DU spectrophotometer at a wavelength of 500 nm.

Production of antiserum to groups A, C, and G. Antiserum for the conjugates and precipitin tests were produced by immunizing white New Zealand rabbits, during a 3-week period, with daily intravenous injections for the first 5 days of each week followed by a 2-day rest period. Dosage consisted of 0.5 ml the first day, 1 ml per day for the remainder of the first week, and, finally, 2 ml per day for the next 10 injections. Rabbits were tested on the seventh day after the last injection. Those found to have a good precipitin titer were bled out the same day, and the serum was collected aseptically and stored in a refrigerator in vaccine bottles. Rabbits with an unsatisfactory titer were discarded, and the immunization schedule was renewed with fresh animals.
Precipitin tests. The strains of streptococci to be tested were grown for 18 to 24 hr in 50-ml centrifuge tubes containing 40 ml of Todd-Hewitt broth. Streptococcal extracts for the precipitin test were prepared by the autoclave method (Rantz and Randall, 1955). Cross-reactions among groups A, C, and G were reduced by adsorbing 2-ml samples of each antiserum for 2 hr in a water bath (37 C) with heat-killed packed sediment from two 40-ml broth cultures of the other two antigens. Additional precipitating antisera for duplicate testing were purchased from Difco. The precipitin tests were performed in capillary tubes (Swift, Wilson, and Lancefield, 1943). Result was read after conjugate removed temperature.

Preparation of buffers. A volume of 500 ml of a 0.5 M carbonate-bicarbonate buffer (pH 9.0) was prepared. Buffered glycerin mounting fluid (pH 8.5) was also prepared by adding 3.5 ml of the carbonate-bicarbonate buffer to 45 ml of chemically pure glycerin. The reaction was checked with hydron paper, and adjustments, when required, were made by the addition of either more glycerin or more buffer. Both buffers were stored in a refrigerator.

Conjugation of antiserum. The volume of antiserum containing 490 mg of protein was determined by the biuret method for each conjugate, and this amount, after adsorption with antigens of the other two groups, was labeled with fluorescein isothiocyanate (Marshall et al., 1958). To eliminate cross-reactions completely (Moody, Baker, and Pittman, 1962), samples of undiluted conjugate and of 1:5 and 1:10 dilutions were set up with PBS (0.01 Molar phosphate-buffered saline, pH 7.2) as the diluent. Each milliliter of all dilutions of conjugate and of 0.05 ml of each of the other two undiluted antisera, and to each dilution was added 0.05 ml of undiluted staphylococcus serum per milliliter of conjugate. The three dilutions of each conjugate, which were actually 1:10, 1:50, and 1:100, were tested with a panel of antigens of groups A, B, C, and G. The conjugates of strains as well as strains identified by the precipitin test and biochemical reactions (Evans, 1944). In addition, a new method of blocking cross-reactions between group A conjugate and groups C and G antigens (Krause, 1963; Karakawa, Mageau, and Borman, 1964) was evaluated during the final phase of this study. For this evaluation, a sample of group A-variant antiserum, produced in rabbit R1462 with strain A486 variant, was kindly supplied by Rebecca C. Lancefield, The Rockefeller Institute, New York, N.Y. Preparation consisted of adding 0.1 ml of group A-variant antiserum per milliliter of various dilutions of adsorbed group A conjugate. Finally, to compare the results of the conjugates produced in this laboratory with those available on the market, commercially prepared group A conjugate was purchased from Difco and BBL.

Media. In addition to the 50-ml centrifuge tubes containing 40 ml of Todd-Hewitt broth, already described, 10-ml samples were distributed in 18-ml screw-cap culture tubes. Two types of blood-agar base medium, Tryptose Blood Agar Base and tryptic soy-agar (Difco), were prepared. Both media contained 7% defibrinated sheep blood.

Throat cultures. Four series of throat cultures were collected and examined. The first group was composed of 417 plates received from the Bacteriology Laboratory, Walter Reed General Hospital, Washington, D.C. Since these specimens came in at the beginning of the study, they were lyophilized and examined later in the phase of the study concerned with development of a simple procedure for grouping β-hemolytic streptococci. The impure Todd-Hewitt broth cultures had been developed, an additional 113 plates, positive for β-hemolytic streptococci, were received from Walter Reed, and 111 plates were received from the Bacteriology Laboratory, U.S. Air Force Hospital Andrews, Andrews Air Force Base, Md. Both of these groups were subcultured and examined as described below. As a final check on the complete procedure, dry throat swabs (Williams, 1958) were collected from 190 children with symptoms of an upper respiratory infection who were examined at the Walter Reed Pediatric Clinic. The delay in receiving the dry swabs was never greater than 4 hr. Upon arrival in this laboratory, each swab was dipped into a tube of Todd-Hewitt broth, spread against the wall to remove excess fluid, and then streaked over one-fifth of the surface of each of two blood-agar plates (one of each type). Streaking for good isolation was completed with a platinum loop, and the plates were incubated in a candle jar overnight at 37 C (Fry, 1933; Fuller and Muxted, 1939). The plates were incubated 0.05 ml of undiluted staphylococcus serum per milliliter of conjugate. The three dilutions of each conjugate, which were actually 1:10, 1:50, and 1:100, were tested with a panel of antigens of groups A, B, C, and G. The conjugates of strains as well as strains identified by the precipitin test and biochemical reactions (Evans, 1944). In addition, a new method of blocking cross-reactions between group A conjugate and groups C and G antigens (Krause, 1963; Karakawa, Mageau, and Borman, 1964) was evaluated during the final phase of this study. For this evaluation, a sample of group A-variant antiserum, produced in rabbit R1462 with strain A486 variant, was kindly supplied by Rebecca C. Lancefield, The Rockefeller Institute, New York, N.Y. Preparation consisted of adding 0.1 ml of group A-variant antiserum per milliliter of various dilutions of adsorbed group A conjugate. Finally, to compare the results of the conjugates produced in this laboratory with those available on the market, commercially prepared group A conjugate was purchased from Difco and BBL.

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Immunofluorescent reaction. After drying, one
TABLE 1. Immunofluorescent reactions of A-variant-inhibited group A conjugate with 40 known β-hemolytic strains of streptococci

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Strains</th>
<th>Conjugate dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:10</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
<td>20*</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>6†</td>
</tr>
<tr>
<td>G</td>
<td>8</td>
<td>4†</td>
</tr>
</tbody>
</table>

* Numbers indicate the number of strains that gave a positive immunofluorescent reaction.
† Conjugate A152 gave negative reactions with groups C and G antigens in 1:10 dilution; A32 was positive in this dilution.

A drop of the Air Force Institute of Pathology antiserum of group A conjugate was added to the smear on the left, and one drop of the commercial conjugate was added to the smear on the right. The drops were carefully spread with applicator sticks, and the slides incubated in a moist dish at room temperature for 30 min. The excess conjugate was gently floated off with PBS from a wash bottle, and the slides were washed in PBS on a slide-rotating machine for 10 min. After washing, slides were gently blotted, one drop of buffered glyc erin and a cover slip were added over each smear, and the slide was examined with a fluorescence microscope. Contact between the slide and condenser was made with a drop of nonfluorescent immersion oil. The assembly used throughout the study consisted of the Zeiss Universal fluorescence microscope, an Osram HBO-200 mercury vapor lamp, Schott BG12 (3 mm) exciter filter, and OG1 (2 mm) barrier filter (Mellors, 1955). Reactions in which the antigen was colorless-to-green, but without fluorescence, were interpreted as negative. The high group specificity of the antisera permitted interpretation of all other reactions as positive.

Controls. For controls, four slides were set up at the beginning of each run. Slides 1 and 2 were smears of known group A, and slides 3 and 4 were smears of known group C and group G, respectively. To slide 2 was added one drop of unconjugated group A antiserum diluted to the same strength as the group A conjugate. After a 5-min incubation period at room temperature, the excess antiserum was floated off slide 2 with PBS, and the four slides were incubated with group A conjugate for 30 min. When only slide 1 demonstrated fluorescence, the reagents were adjudged accurate. Finally, the results obtained by immunofluorescence were checked the next day with the precipitin test.

Results

Response of rabbits to immunization. Since the immunofluorescent reaction is the final arbiter of the adequacy of the response of a rabbit to immunization (Karakawa, Borman, and McFar-land, 1964), test bleeding was confined to performing precipitin tests only on undiluted samples. Any antiserum that gave a good reaction could usually be depended on to give reliable results as a conjugate in a dilution of at least 1:50. Antisera were produced on three different occasions, and all except 1 of the 18 rabbits used developed satisfactory titers.

Conjugates. The addition of unconjugated antiserum of two of the groups to the conjugated antisem of the third group eliminated cross-reactions in all dilutions of 1:50 or higher. The use of group A-variant antiserum to inhibit cross-reactions of group A conjugates with groups C and G antigens (Table 1) gave excellent results. In fact, this one-step method produced such effective group specificity (Fig. 2 and 5) that even the 1:10 dilution of some group A conjugates gave negative reactions with groups C and G antigens.

Isolation of β-hemolytic streptococci. During the pediatric survey, β-hemolytic streptococci were isolated from 52 (27.3%) of the 190 children. Comparison of the results of the blood plates with those of the duplicate Todd-Hewitt broth tubes showed the latter medium to be unsatisfactory for enhancement of growth of β-hemolytic streptococci in mixed flora from throat swabs when the original inoculum of β-hemolytic streptococci is small. This was repeatedly corroborated during the survey. The plates inoculated from the Todd-Hewitt broth tubes after 4 to 16 hr of incubation developed no β-hemolytic colonies when the original blood plates contained only a few colonies. On the other hand, when approximately 10% of the colonies on the original plate were β-hemolytic, the plate inoculated from the duplicate Todd-Hewitt broth developed β-hemolytic colonies. In addition, strains of group C which produced pinpoint colonies with a zone of hemolysis about 1 mm in diameter could not be isolated from Todd-Hewitt broth, even though growth on the original plate was luxuriant. Both types of blood-agar media were satisfactory, but strains of α streptococci did not simulate β-hemolytic streptococci on tryptic soy-agar as often as on Tryptose Blood-Agar.

Identification of group A streptococci. After investigating the problems of immunofluorescence by examining most of the 417 specimens collected during the first phase of the study, a technique was developed that gave accurate results in comparison with the precipitin test. Although the agreement between the immunofluorescence results and the precipitin results was excellent (Tables 2 and 3), this would not have been true if each distinct type of β-hemolytic colony had not been separately inoculated for preparation of precipitinogen. In some cases, all of two or three dif-
different colonial types proved to be of the same group (Williams, 1958) and in agreement, with the immunofluorescence. In other instances, one colonial type proved to be of the same group as that identified with immunofluorescence, whereas one or more additional colonial types proved to be nonreactive. On the other hand, mixed cultures presented no problem in the identification of specific antigens with immunofluorescence. Figure 4, a mixture of groups A, C, and G, is an example of this specificity. Positive reactions always gave a high degree of fluorescence (Fig. 1, 4, 5, and 6). Negative reactions (Fig. 2 and 3) usually exhibited only a dull-green color except in instances of a mass of organisms. When this occurred, study of the smear with the 40 X objective quickly demonstrated that it was not true fluorescence. Genuinely fluorescent organisms always continued to glow even when viewed with objectives of much higher power than the 16 X. Very little light is reflected by negative reactions; consequently, they are difficult to photograph.

As has been stated previously, the smear on the right side of the slide always received one drop of one of the two commercial group A conjugates. The results were never at variance with the group A conjugates produced here, but the degree of fluorescence was less. The commercial antisera which remained after storage for 6 months showed a definite decrease in activity, however.

**Discussion**

During the early phase of this study, the immunofluorescent method of grouping β-hemolytic streptococci was discussed with several directors of large clinical laboratories. They expressed very little interest in the procedure for two reasons: (i) It is too difficult to perform accurately. (ii) It is not worth the extra work, since 95% of the β-hemolytic streptococci isolated from throat cultures are group A organisms. I trust that the results of this study will be an answer to both criticisms. In addition, a search of the literature revealed one important source for the second statement (Burrows, 1963). It appears that it is based upon a misinterpretation of Evans' (1944) table, "Incidence of streptococci of groups A and C in human infections." Evans (1944) reported that group A was the etiological agent in 96.9% of the 552 cases that she studied. Without embelloring the point, it is obvious that this table does not refer to the isolation and group distribution of β-hemolytic streptococci in man.

There is general agreement on the importance of group A streptococci in the etiology of rheumatic fever and acute glomerulonephritis. Accordingly, physicians are desirous of quickly treating cases of upper respiratory infection due to group A streptococci. Since, however, most clinical laboratories simply report "β-hemolytic streptococci found," they have to base their treatment completely on their clinical judgment. The question that now arises is, how often are patients with upper respiratory infections being treated with penicillin unnecessarily? In the two surveys conducted for this study, at least one patient in every six from whom β-hemolytic streptococci were isolated did not have group A streptococci.

There are similar reports in the current literature (Hunter, Blair, and Rust, 1962; Rauch and Rantz, 1962; Jablon and Brust, 1963). Of particular interest is the report by Hunter et al. (1962) of an outbreak of acute upper respiratory infection in a military recruit population in which there were 597 hospitalized cases. Single throat cultures showed β-hemolytic streptococci present in 253 cases (39.8%); only 98 (41.7%) of those from whom β-hemolytic streptococci were isolated had infection with group A streptococci, however. Treatment of patients in this outbreak

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**Table 2. Immunofluorescent and precipitin reactions of β-hemolytic streptococcal strains from 224 cultures previously reported positive**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunofluorescence</th>
<th>Precipitin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Per cent</td>
</tr>
<tr>
<td>A</td>
<td>190</td>
<td>84.8</td>
</tr>
<tr>
<td>C</td>
<td>16</td>
<td>7.2</td>
</tr>
<tr>
<td>G</td>
<td>11</td>
<td>4.9</td>
</tr>
<tr>
<td>NR*</td>
<td>7</td>
<td>3.1</td>
</tr>
<tr>
<td>Total</td>
<td>224</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* No reaction.

**Table 3. Immunofluorescent and precipitin reactions of β-hemolytic strains of streptococci isolated from 83 children**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunofluorescence</th>
<th>Precipitin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Per cent</td>
</tr>
<tr>
<td>A</td>
<td>43</td>
<td>82.7</td>
</tr>
<tr>
<td>B*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>1.9</td>
</tr>
<tr>
<td>F*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G</td>
<td>3</td>
<td>5.7</td>
</tr>
<tr>
<td>NR†</td>
<td>5</td>
<td>9.7</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Fluorescein-labeled antisera not available.
† No reaction.
FIG. 1. Fluorescence of group A streptococci in the presence of group A conjugate (three-step inhibition). X400.

FIG. 2. Fluorescence absent with group G chains in the presence of group A conjugate (one-step inhibition). X400.

FIG. 3. Fluorescence absent with group C chains in the presence of group A conjugate (three-step inhibition). X400.


FIG. 5. Fluorescence of group A streptococci in the presence of group A conjugate (one-step inhibition). X400.

FIG. 6. Fluorescence of group C streptococci in the presence of group C conjugate (three-step inhibition). X400.
with penicillin, solely on the basis that presence of β-hemolytic streptococci meant group A, would have been unnecessary in three of five cases. Under such conditions, a significant amount of erroneous information may be given to the physician when neither the precipitin test nor immunofluorescence is used for verification. Of the two methods, immunofluorescence is the one of choice. It requires a significantly shorter period (a maximum of 24 hr) for completion than does the precipitin test. The bacitracin disc method for identifying group A streptococci, which was first reported by Maxted (1953), was not included in this study, because it usually requires 48 hr or more for completion. In addition, Estela and Shuey (1963), who also recommended immunofluorescence as the method of choice in the identification of group A streptococci, found a 10% error in the bacitracin disc method.

It will be noted that whole serum, rather than globulin, was used to prepare the conjugates, and that group specificity was obtained through adsorption and inhibition of cross-reacting antibodies. In spite of this departure from the usual method of conjugating globulin precipitated with ammonium sulfate, the results obtained were in agreement with the precipitin test and the commercial conjugates (globulin preparations) from two separate sources. In view of these results, it appears that conjugation of whole serum, which avoids the technical difficulties of ammonium sulfate precipitation, is a satisfactory method at least for group A, C, and G streptococci.

As reported in Krause's (1963) recent review, the major extractable carbohydrate in the cell walls of group A, C, and G streptococci is rhamnose, but practically all of the extractable carbohydrate in the cell wall of group A-variant streptococci is rhamnose. This preponderance of rhamnose, with only a small amount of amino sugar, in the wall of group A-variant streptococci, and its presence to a lesser degree, with an appreciable amount of amino sugars, in the walls of groups A, C, and G, probably explains the excellent results obtained with the use of group A-variant antisera in inhibiting the cross-reactions of group A conjugate. This is in agreement with the prediction of Heidelberger (1964) that cross reactivity of two molecular species with an antibody to one of them may reasonably be expected if the two molecules contain multiples of a common chemical grouping.

Although this is in agreement with the data I have presented on the use of group A-variant antisera to inhibit cross-reactions of group A conjugate, it is also a possible basis for hesitancy in introducing the immunofluorescent method for identification of bacteria. In practice, however, cross-reactions of streptococcal conjugates with other antigens have not been a problem. This is due to observance of a simple rule: to be identified as a given organism, a fluorescent body must present a compatible morphological pattern.

The results reported here and those from field trials still in progress in this laboratory indicate that introduction of the immunofluorescent method for grouping β-hemolytic streptococci in the clinical laboratory, especially the larger ones, is feasible. Until adequate experience is gained, however, the confirmatory precipitin test should also be performed. When this point has been reached, the routine precipitin confirmation may be discontinued.

Acknowledgment

I am indebted to Elmer F. Chaffee and Chauncey W. Smith, Immunology and Bacteriology Branch, for their valuable comments, and to Roger M. Cole, National Institute of Allergy and Infectious Diseases, and Rebecca C. Lancefield, The Rockefeller Institute, for the strains and antiserum they provided. I am especially indebted to John P. Fairchild, Pediatric Service, John T. Maloney, Bacteriology Laboratory, Walter Reed General Hospital, and to William H. Hill, Bacteriology Laboratory, U.S. Air Force Hospital Andrews, without whose cooperation in providing throat cultures this study would have been impossible.

Literature Cited


