NON-BETA-HEMOLYTIC GROUP M-REACTING STREPTOCOCCI OF HUMAN ORIGIN

DAVID RIFKIND AND ROGER M. COLE

Laboratory of Clinical Investigations and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Received for publication February 1, 1962

ABSTRACT

RIFKIND, DAVID (National Institutes of Health, Bethesda, Md.) AND ROGER M. COLE. Non-beta-hemolytic group M-reacting streptococci of human origin. *J. Bacteriol.* **84**:163-168. 1962.—In 8 years, 14 strains of alpha- and gamma-hemolytic streptococci, reacting only with group M antiserum, were isolated from a variety of human sources. Two alpha-hemolytic strains from the blood of endocarditis patients were compared biochemically and immunologically with the two original canine beta-hemolytic strains of Fry (ATCC 9934 and 9935). The human strains do not produce ammonia or ferment glycogen, whereas the animal strains do. The animal strains share two trypsin-labile antigens with the human strains, and one of them (ATCC 9935) also shares at least four trypsin-stable antigens with the human strains. The other strain of Fry (ATCC 9934) appears to lack these trypsin-stable antigens. These results indicate that the human strains correspond to those designated biotype I whereas ATCC 9935 belongs to biotype II and ATCC 9934 to biotype III.

The nature of the group M antigen is undefined, but the gel-diffusion methods employed in this work suggest that several antigens may be responsible for precipitates seen as positive reactions to group M antisera when testing is done by the usual methods in tubes.

Beta-hemolytic streptococci assigned to Lancefield group M were originally isolated by Fry (1941) from the nasopharynx and genito-urinary tract of dogs. A group M streptococcus of unstated origin, which regularly produced alpha-hemolytic variants otherwise identical with the parent strain, was described by Brooke (1950). Skadhauge and Perch (1959) investigated ten strains of alpha-hemolytic streptococci reacting with group M antisera, which were isolated from the nasopharynx of normal humans and from the

blood of four patients with subacute bacterial endocarditis. These strains could be differentiated both serologically and biochemically from canine beta-hemolytic group M streptococci.

Over an 8-year period, 14 strains of alpha- and gamma-hemolytic (i.e., nonhemolytic) group M-reacting streptococci were isolated from a variety of sources, including the nasopharynx, vagina, abscesses, and blood. The present report is concerned with biochemical and immunological studies of two of these strains, each from a patient with subacute bacterial endocarditis, which have been shown to be distinguishable from Fry's original beta-hemolytic group M streptococci.

MATERIALS AND METHODS

Streptococcal strains. Two beta-hemolytic group M streptococci (American Type Culture Collection strains 9934 and 9935; Fry's original group M strains), two alpha-hemolytic group M-reacting strains from subacute bacterial endocarditis (N.S. and W.S.), *Streptococcus mitis* (ATCC 9811), and *Streptococcus salivarius* (ATCC 9759), were studied.

Biochemical characteristics. Hemolysis was observed on 5% human, rabbit, and sheep blood agar plates which were streaked and stabbed and incubated at 35 C for 1 to 2 days.

Fermentation reactions were determined in Cystine Trypticase agar (Baltimore Biological Laboratories, Baltimore, Md.) containing 1% carbohydrate. Tubes were incubated and observed daily for 1 week.

Ammonia production was determined by Nessler's reagent after 4 days of incubation in Todd-Hewitt broth (Difco) containing 4% peptone.

Serology. For preliminary grouping, all streptococci were tested by the capillary tube precipitin method (Swift, 1947) using group A, B, C, D, E, G, H, L, and M antisera. The standard group

M antiserum was prepared using a vaccine of ATCC strain 9935.

Antisera were produced in rabbits with a 0.2% formalized vaccine injected intravenously over a period of 3 to 6 weeks. Daily injections were given using 1 ml of a 1% suspension for the first week and a 2.5% suspension thereafter. Where indicated, sera were absorbed twice using one-tenth volume of a 2.5% vaccine suspension.

Acid extracts of the streptococci were prepared following the method of Lancefield (1933). Certain extracts were then trypsinized in the following manner. Extract (4 volumes) was mixed with 1 volume of 1% trypsin (Difco trypsin, 1:200) in 0.85% saline (pH 7.2 to 7.4), incubated in a water bath at 37 C for 1 hr, and then heated at 60 C for 1 hr to inactivate the enzyme.

Extracts were tested by the double-diffusion method of Ouchterlony (1949) using plates prepared with 1% Noble agar (Difco) in 0.85% saline and containing thiomerasol (final concentration, 1:10,000). These plates were incubated in humidified containers at room temperature and observed daily for 1 week.

RESULTS

Source of cultures. During the 8-year period (1952 to 1960), 310 strains of non-beta-hemolytic streptococci of human origin were submitted to this laboratory for identification. Of these a total of 14 was found to react specifically with group M antiserum.

Four strains were isolated from blood cultures, of which two were from patients with subacute bacterial endocarditis, one from a patient with possible subacute bacterial endocarditis, and one from a post-mortem cardiac blood sample in mixed culture with a paracolon species. Two strains were from abscesses in mixed culture with *Staphylococcus aureus* in one case and *Bacteroides* species in the other. One strain was from the discharge from a case of trichomonal vaginitis. Seven strains were from throat culture specimens, which were submitted without clinical diagnoses. Of the

14 strains, 10 were alpha-hemolytic and the other 4 were gamma-hemolytic. No beta-hemolytic streptococci of human origin, reacting with group M antisera, have been identified by this laboratory.

The two endocarditis strains, N.S. and W.S., were available for more detailed biochemical and serological studies, and were compared with the two canine beta-hemolytic group M strains, ATCC 9934 and 9935.

Biochemical reactions. Some biochemical reactions of those strains are shown in Table 1. It is seen that, in addition to hemolysis, the human and animal strains can be differentiated on the basis of ammonia production and glycogen fermentation. The group M streptococci produced ammonia and fermented glycogen, and the endocarditis strains carried out neither of these reactions. Also, starch fermentation by the human strains was delayed 3 to 4 days, in contrast to the animal strains which produced acid promptly within 24 hr.

Serological reactions. Acid extracts of group M streptococci 9934 and 9935 reacted with anti-9935 serum on the basis of at least two common antigens (Fig. 1). In addition, with this antiserum, strain 9935 and endocarditis strains N.S. and W.S. demonstrated antigens in common.

Extracts treated with trypsin, when tested against anti-9935 serum (Fig. 2), revealed that the antigens shared by the two group M strains, 9934 and 9935, were trypsin-labile, and those shared by strains 9935 and endocarditis strains N.S. and W.S. were trypsin-stable. The composition of this trypsin-stable complex is better analyzed by using dilutions of trypsinized extracts with endocarditis strain W.S. antiserum (Fig. 3), where at least four antigens shared by these three streptococcal strains are revealed.

Antigens are shared by both group M strains, both endocarditis strains, and *S. mitis* and *S. salivarius* as shown with anti-N.S. serum (Fig. 4). These antigens were found to be trypsin labile. It will be noted that antibodies to the trypsin-

TABLE 1. *Biochemical reactions of group M-reacting streptococci*

Strain	Origin	Hemolysis	Ammonia produced	Glucose (final pH)	Starch	Glycogen	Inulin	Salicin	Esculin
W.S.	Human	Alpha	—	5.05	+	—	—	—	—
N.S.	Human	Alpha	—	5.50	+	—	—	—	—
9935	Animal	Beta	+	5.95	+	+	—	—	—
9934	Animal	Beta	+	5.75	+	+	+	—	—

FIG. 1. Reactions of streptococcal extracts (in side wells numbered 1 through 6 starting from top clock-wise) with antiserum (in center well). Acid extracts: (1) 9934; (2) 9935; (3) N.S.; (4) W. S.; (5) *Streptococcus mitis*; (6) *S. salivarius*. Antiserum: 9935.

FIG. 2. Reactions of trypsinized acid extracts, 1 through 6 (as in Fig. 1), with antiserum 9935.

FIG. 3. Reactions of trypsinized acid extracts with antiserum W.S. Extracts: (1) W.S., diluted 1:8; (2) 9935, diluted 1:4; (3) N.S., diluted 1:4; (4) W.S., diluted 1:4; (5) 9935, diluted 1:8; (6) N.S., diluted 1:8.

FIG. 4. Reactions of acid extracts, 1 through 6 (as in Fig. 1), with antiserum N.S.

stable complex described above did not appear in this particular antiserum.

Using 9935, N.S., and W. S. antisera it was not possible to show a trypsin-stable antigen in the second group M strain 9934. Further, no trypsin-stable antigen could be demonstrated using homologous antisera prepared in rabbits immunized over a 3-week period with 1 ml of a 1 to 2.5% suspension of vaccine.

DISCUSSION

The Lancefield group M as originally described by Fry was composed of beta-hemolytic streptococci isolated from dogs. To define the relationship of the human non-beta-hemolytic group M-reacting strains reported here with the canine streptococci, an attempt was made first to define more precisely the group M antigen using the agar-diffusion method. Both beta-hemolytic strains studied were shown to have in common at least two trypsin-labile antigens not present in the alpha-hemolytic endocarditis strains. In addition one of the two beta-hemolytic strains (9935) was found to share at least four trypsin-stable antigens with both endocarditis strains. It is not clear, however, whether group specificity resides in the trypsin-labile or in the trypsin-stable components. If the group M were defined on the basis of one or more trypsin-labile (presumably protein) antigens, this would not seem to be in conformity with the other streptococcal groups, which are characterized by specific "C" polysaccharides (Niven, 1957). Conversely, the trypsin-stable bands, although probably more closely related to the usual group-specific polysaccharides, were found in only one of the two group M strains tested.

Although group M strain 9934 does not appear to share trypsin-stable antigens with 9935, it is possible that the former strain possesses them or still another complex of such antigens. The failure to demonstrate these antigens with homologous antisera may simply indicate that the course of immunization was too brief.

The present data then are not sufficient to define the group M antigen and thus to determine whether the reaction of these alpha-hemolytic endocarditis strains with M antisera indicates the presence of the group-specific antigen. The multiplicity of trypsin-stable antigens present in acid extracts of at least one M streptococcus, however, suggests that the group M "antigen" may actually be composed of a number of antigens, a fact not discernible by the ordinary methods of tube-precipitin testing. Further, it would be of interest to determine whether a similar situation might be obtained in some of the other streptococcal groups, particularly group A, as suggested by the work of Pierce (1959).

The biochemical reactions of the two alpha-hemolytic group M-reacting strains are in general accord with those of the human isolates reported by Skadhauge and Perch (1959), and designated biotype I (Table 2). These workers reported that the human strains did not ferment 'starch' although they employed glycogen for these determinations, apparently on the assumption that the results would be identical. The two human strains reported here (Table 1) did not ferment glycogen. However, they did ferment starch after a delay of several days. Beta-hemolytic group M strains 9935 and 9934 correspond biochemically to their biotypes II and III, respectively. In addition, our serological studies confirm and extend their findings that biotypes I and II share a heat-stable antigen resistant to proteolytic enzymes, and biotypes II and III share a heat-labile antigen damaged by trypsin.

It seems likely that two types of non-beta hemolytic streptococci reacting with group M antisera may occur. The first is represented by the endocarditis strains reported here and by the human biotype I strains (Skadhauge and Perch, 1959). These strains are distinguishable both biochemically and serologically from the canine beta-hemolytic strains. The second is represented by the alpha-hemolytic variants of a classical beta-hemolytic strain, which were identical in

TABLE 2. Classification of group M-reacting streptococci*

Biotype	Origin	Hemolysis	Arginine hydrolyzed	Glucose (final pH)	Glycogen	Inulin	Salicin	Esculin	No. of strains
I	Human	Alpha	—	4.62–5.25	—	—	—	—	10
II	Animal	Beta	+	6.26–7.20	+	—	—	—	7
III	Animal	Beta	+	5.96–6.74	±	±	±	±	3

* Adapted from Skadhauge and Perch (1959). Symbol: ± = variation among strains.

biochemical and serological characteristics to the parent organism as reported by Brooke (1950).

With the wider application of group-specific antisera in the identification of non-beta-hemolytic streptococci, it may be anticipated that study of additional strains of these human group M-reacting streptococci and of the beta-hemolytic animal forms will aid in defining their taxonomic relationship.

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