CLASSIFICATION OF THE HEMOLYTIC STREPTOCOCCI BY THE PRECIPITIN REACTION.

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Following the demonstration by Schottmüller that a useful classification of the streptococci could be made in accordance with their cultural characteristics upon blood agar, a large number of studies have dealt with that family which is defined by the power of its members to produce clear zones of hemolysis surrounding their colonies. That this clinically and pathologically important group should arouse great interest was natural, and that attempts should be made further to analyze its biological properties and if possible to correlate them with the disease pictures produced was obvious.

The earlier studies of this group were concerned largely with morphological, cultural, and fermentative properties. The two former were soon abandoned as means of subclassification, as showing too great variability for practical purposes. Lyall (3), introducing a roughly quantitative method for measuring the degrees of hemolysis produced by various strains, studied their fermentation reactions and observed a measure of uniformity among his cocci; 74.9 per cent fermented salicin alone, while 14.1 per cent more were indifferent to the four carbohydrates employed. Shortly thereafter Holman (4), in promulgating his general classification of the streptococcus family, found that the great majority of his hemolytic strains fell into one group (*Streptococcus pyogenes*), but was able to designate seven variant groups in addition. Obviously such methods were unsatisfactory and temporary, based as they were upon the use of an arbitrarily limited number of substrates. Floyd and Wolbach (2) had previously combined the study of fermentation and agglutination reactions, and, while failing to obtain a more satisfactory grouping of the strains, had observed that there was some degree of correlation possible between the results of the two methods.

Employing the complement fixation reaction, Kinsella and Swift (5) found that their twenty-eight hemolytic strains yielded such marked cross-fixation as to lead to the conclusion that all were closely related or even practically identical, and in

addition showed an orderly relationship to certain members of the non-hemolytic family (6). This was disputed by Howell (7), who was able to demonstrate cross-fixation in only 62 per cent of her tests.

Refinements of technique enabled Dochez, Avery, and Lancefield (9), on the basis of their studies of the agglutination and protection reactions, to demonstrate four major groups of hemolytic streptococci, with a small number of variants among which two further groups were later determined. All the mannitol-fermenting strains fell under one head. Further studies by Dochez and Bliss (10), Bliss (11), and Tunnicliff (12) were directed to the problem of establishing the specificity of those strains associated with scarlet fever.

Davis (1) had observed cross-sensitization in the guinea pig among several strains of the organism under consideration, but confusing cross-reactions appeared between this family and the pneumococci.

The precipitin reaction has received scant attention. Barnes (8) attempted with a fair measure of success to correlate the results of this reaction with those of fermentation, but scrutiny of his results engenders doubts as to the exact nature of the phenomena which he was observing. In view of the striking neglect of this phase of the question, it seemed of interest to study by means of the precipitin reaction a number of strains of hemolytic streptococci, employing as antigens extracts similar to the "residue antigens" described by Zinsser and Parker (13). Similar or identical bacterial products have recently been shown by Avery and Heidelberger (14) to be type-specific within the pneumococcus family. The entire absence of cross-reaction suggests that in these "residues" we possess excellent materials for the group differentiation of bacteria.

Methods.

1. Table I presents the thirty-two strains employed, with reference to their sources and properties.

An effort was made to secure a fairly representative collection of organisms. In every case the cultures were plated out at least twice upon blood agar, and each time single colonies were picked for isolation. Bile solubility tests were made by mixing 0.2 cc. of sterile ox bile with 0.8 cc. of 24 hour plain broth culture, and incubating in the water bath at 37° for 2 hours. For testing the effect upon red cells, 0.5 cc. of a 24 hour culture in plain broth was mixed in a small tube with an equal volume of a 5 per cent suspension of sheep red cells in saline, and the mixture was incubated in the water bath at 37° for 1 hour.

TABLE I.

Sources and Properties of Strains of Hemolytic Streptococci Employed in This Study.

		Bile.	5 per cent sheep cells.
R80	Rabbit, spontaneous infection.	Insoluble.	Prompt hemolysis.
S1	Rectum, proctitis.	"	" "
S2	Throat, follicular tonsillitis.	"	" "
S3	Chest fluid, pyopneumothorax.	"	" "
S4	Throat, follicular tonsillitis.	"	" "
S5	Blood culture, wound infection, septicemia.	"	" "
S6	Throat, scarlet fever.	"	" "
S6K	Right kidney (necropsy), scarlet fever.	"	" "
S7	Uterus (necropsy), puerperal sepsis, peritonitis.	"	" "
S8	Pus, empyema.	"	" "
S9	Throat, suspected scarlet fever.	"	" "
S10	" scarlet fever.	"	" "
S11	" peritonsillar abscess.	"	" "
S12	" follicular tonsillitis.	"	" "
S13	Sputum, bronchopneumonia.	"	" "
S14	Axillary gland (necropsy), empyema, Hodgkin's disease, septicemia.	"	" "
S15	Throat, follicular tonsillitis.	"	" "
S16	Sputum, lobar pneumonia.	"	" "
S17	Pus, mastoiditis.	"	" "
S18	Blood culture, puerperal sepsis.	"	" "
S19	Heart's blood (necropsy), thrombophlebitis, terminal septicemia.	"	" "
S20	Heart's blood (necropsy), pneumothorax, hemopericardium.	"	" "
S21	Pus, mastoiditis.	"	" "
S22	Throat, scarlet fever.	"	" "
S23	" " "	"	" "
S24	" " "	"	" "
S60	" measles.	"	" "
S107	Sputum, "	"	" "
S139	Throat, bronchopneumonia.	"	" "
S177	" follicular tonsillitis.	"	" "
S212	" " "	"	" "
S222	" " "	"	" "

2. Bacterial extracts for use as antigens in the precipitin reaction were prepared as follows:

The organisms from a 24 hour culture in 1 per cent glucose beef infusion broth were sedimented by centrifugation, washed once with physiological saline, and recentrifuged. The packed organisms were desiccated overnight *in vacuo* over phosphoric anhydride; the dry residue was finely ground in a small mortar, and was weighed to the nearest milligram. It was then extracted with 0.8 per cent antiformin, 0.5 cc. per mg. of dried organisms, in the water bath at 56°, for 10 minutes, being shaken vigorously two or three times during the extraction. The suspension at the end of the designated time was treated with a 5 per cent solution of sodium thiosulfate, 3 drops being used per cc. of diluted antiformin; it was then rapidly cooled to room temperature under the tap. $\frac{N}{5}$ sulfuric acid was added slowly and with gentle agitation until a heavy flocculent precipitate appeared. At this point the mixture was tested with litmus paper, and, if necessary, further acid was added drop by drop until a *faint* acid reaction appeared. The liquid was then centrifuged for a few minutes at moderate speed, and the clear supernatant solution was removed by decantation. This solution was brought to the neutral point with a drop or two of $\frac{N}{5}$ sodium hydroxide, and saline containing 0.5 per cent phenol was added until the final volume was such that 1 cc. represented 1 mg. of the original desiccated organism. This method was strictly adhered to, in the hope that the final solutions would be comparable in a roughly quantitative sense.

As different lots of antiformin have been found to vary appreciably in their available chlorine content, and as the preparation of a satisfactory extract is more dependent upon this factor than upon that of alkalinity, it was found advisable to titrate each new lot in order to obtain the best results. 10 cc. of the diluted antiformin were measured into a small flask, and to this were added 0.25 gm. of solid KI and 1 cc. of strong acetic acid. The liberated iodine was titrated with $\frac{N}{10}$ sodium thiosulfate, with weak starch solution as indicator. If the available chlorine was found to be approximately equivalent to 1.7 cc. of the standard thiosulfate, the dilution of antiformin was considered satisfactory for use.[1]

3. Rabbits were used for the preparation of antisera. Immunization was effected by the intravenous injection of whole 18 hour cultures in plain meat infusion broth of pH 7.6, sterilized by heating in the water bath at 56° for 1 hour. After considerable experimentation, the following method was adopted as yielding the most satisfactory results.

[1] The author is greatly indebted to Dr. A. P. Briggs for suggesting this method of titration.

On 4 successive days the animals were given injections of 1, 2, 5, and 10 cc. respectively. After a lapse of 5 days followed a further course of four doses, the first two of which were of 10 and 20 cc. respectively. The third and fourth doses consisted of the organisms sedimented by centrifugation from 50 cc. of plain broth culture and resuspended in 10 cc. of the supernatant liquid. After the further lapse of 1 week, the animals received three injections, each consisting of the organisms sedimented from 100 cc. of broth culture and resuspended in 10 cc. of the supernatant liquid. Trial bleedings were made 1 week following the last dose. If the precipitin content of the serum was not satisfactory, the last series of three injections was repeated once or twice, at weekly intervals, and the animals were bled 1 week following the last injection. In every case the rabbits responded by the production of precipitating antibodies, although there was considerable individual variation in the potency of the sera obtained. In all, nine satisfactory sera were prepared.

4. Precipitation tests were performed as follows:

0.2 cc. of the serum at hand was placed into each of a number of small tubes, and to each tube was added 0.2 cc. of antigen prepared as above outlined. The contents were mixed by gentle shaking. Positive results were denoted by the immediate clouding of the mixture. With the most potent sera, rapid flocculation appeared, and within an hour at room temperature the lower third of the tube was occuped by a heavy, coarse precipitate. The tubes were allowed to stand at room temperature for several hours, and were then placed in the ice box overnight before the final reading.

RESULTS.

These are presented in summary in Table II.

In every case there was made a series of control tests, with 0.2 cc. of normal rabbit serum in place of the immune serum. Furthermore, each immune serum was tested with antigens prepared as above from each of sixteen strains of non-hemolytic streptococci. In no instance was there any indication of cross-precipitation or of any non-specific flocculation.

Of the thirty-two strains of true hemolytic streptococci employed in this study, thirty-one gave immediate, constant, and practically uniform precipitation with each of the immune sera at hand. After standing several hours in the ice box, the precipitate was found to consist either of definite coarse flakes which could easily be observed by gentle agitation of the tube, or else of a densely matted mass which could not be broken up even by violent shaking. One strain con-

TABLE II.

Hemolytic Strains.

	R80	S1	S2	S3	S4	S5	S6	S6K	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S60	S107	S139	S177	S212	S222
Serum R80....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" S1.....	3	3	3	3	3	3	3	3	2	0	2	3	3	3	3	3	3	3	3	3	2	2	2	3	3	3	3	3	3	2	3	3
" S2.....	4	4	4	4	4	4	4	4	4	0	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	3	4	4	4	4	4	4
" S3.....	4	4	4	4	4	4	4	4	4	0	4	3	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4
" S7.....	3	3	2	3	3	3	3	3	2	0	3	3	3	3	3	3	3	3	3	3	3	2	2	3	3	2	3	3	3	3	3	2
" S16.....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	2	3	3	3	3	3	3
" S22.....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3	3
" S23.....	3	3	3	3	3	3	3	3	3	0	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" S139....	3	3	2	2	2	2	2	2	2	0	2	3	3	3	2	2	3	2	2	2	2	2	2	2	2	3	2	2	2	2	2	2
Normal rabbit serum.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Non-Hemolytic Strains.

	A4	A49	A135	A179	B1	2B	B3	B4	C1	2C	C3	2D	38D	MB	R	W72
Serum R80.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S1.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S2.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S3.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S7.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S16.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S22.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S23.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" S139.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Normal rabbit serum.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4 indicates heavy, matted precipitate, not fragmented upon agitation; 3, the same but less in amount; 2, flocculent precipitate, fragmented upon agitation; 0, no precipitate.

stantly failed to give positive results even with the strongest sera. Further study of this organism has shown it to be very weak antigenically, and only with difficulty could antisera to it be produced. Such sera yielded weak precipitation and complement fixation reactions with antigens made from the homologous organism, but were entirely devoid of any cross-reaction whatsoever.

DISCUSSION.

This study indicates that on the whole the hemolytic streptococci form one homogeneous group, so far as may be concluded from their property of yielding a common precipitating substance. The existence of variant strains lacking this property is indicated, but undoubtedly they are greatly in the minority. This uniformity is strikingly in contrast with the properties of the non-hemolytic family, which, as will be shown elsewhere, are quite heterogeneous when studied by the same method. The entire absence of cross-reaction between the two groups stands in sharp contrast to the results which have been obtained by means of the complement fixation reaction, in which were used as antigens complete solutions of streptococci in stronger antiformin (6). The solutions prepared as above described are practically free from protein, and it is without doubt this feature which is accountable for their type specificity. Avery and Heidelberger (14) have shown that the protein of the pneumococcus is specific for species but not for type, and observations indicating that the same statement holds true for the streptococci will be published in the near future.

No attempt has been made to study the present series by agglutination, protection, or fermentation reactions. These have been so thoroughly investigated by others that it has seemed unnecessary to to resort to them. There is no reason to question the existence of subgroups within the family of hemolytic streptococci; indeed, a parallel case is afforded by the existence of subtypes of Pneumococcus Type II, all yielding good precipitation reactions with the standard type serum.

SUMMARY AND CONCLUSIONS.

A method is described for the preparation from hemolytic streptococci of extracts containing a soluble precipitating substance.

By the use of such extracts, it is shown that nearly all members of this family yield a common precipitable substance.

So far, therefore, as concerns the precipitin reaction, the hemolytic streptococci form a practically homogeneous group.

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