PRECIPITATION AND COMPLEMENT FIXATION REACTIONS WITH RESIDUE ANTIGENS IN THE NON-HEMOLYTIC STREPTOCOCCUS GROUP.

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The orderly classification of non-hemolytic streptococci has met with the same difficulties as has that of the hemolytic group. Earlier studies failed entirely to recognize the distinction based upon the action of the organisms upon red cells, although this had been clearly pointed out as early as 1903. Later classifications based upon erythrocyte and fermentation reactions (1–3) were arbitrary, but were made to serve as a fair basis for the comparison of different strains. The agglutination and agglutinin absorption methods were found roughly to confirm them, but clear-cut results were not secured (4–7). The complement fixation reaction, as employed by Floyd and Wolbach (4), failed to yield additional information. Using the clear centrifugate from broth cultures, Barnes (8) studied flocculation reactions in immune sera, but failed to clarify the subject perceptibly. His results were later shown by Krumwiede and Valentine to have been in reality thread reactions (9).

Kinsella and Swift (10), employing antiformin antigens, showed by means of complement fixation that an inverse ratio exists between fixability of antigen and fixation range of corresponding antiserum, and were accordingly able to separate their organisms into two main groups, with a few connecting strains. Kinsella later (11) showed that one of these groups showed pronounced cross-fixation with the hemolytic family, whereas the other lacked this property entirely. Howell (12) was unable to confirm these results *in toto*, although she found a certain amount of irregular cross-fixation among her organisms.

In view of the striking neglect of the precipitin reaction, it seemed of interest to study by this method a few strains of non-hemolytic streptococci, with extracts similar to the "residue antigens" described by Zinsser and Parker (13) used as antigens. Similar or identical bacterial products have recently been shown by Avery and Heidelberger (14) to be type-specific within the pneumococcus family. As the application of this method to the non-hemolytic streptococci is more difficult than to the hemolytic family, it was found desirable in every instance to check the results by means of the reaction of complement fixation.

Methods.

1. Table I presents the twelve strains employed, with reference to their sources and properties. 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 plain broth culture was mixed in a small tube with an equal volume of a 5 per cent suspension of washed sheep erythrocytes in saline, and the mixture was incubated in the water bath at 37° for 1 hour.

2. The preparation of the bacterial antigens has been previously described (15), and it is only necessary here to state that the method as outlined was rigidly adhered to.

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. The animals were at first given successive doses of 10 cc. each at 4 day intervals. After ten such injections, trial bleedings were made, and it was found that some of the sera were already rich in complement-fixing and precipitating antibodies. The animals yielding inactive or faintly active sera were given a further course of four injections at 4 day intervals, each dose consisting of the organisms sedimented by centrifugation from 50 cc. of plain broth culture and resuspended in 10 cc. of the supernatant liquid. After the lapse of 4 or 5 days, several of the animals were given a further similar course, each dose consisting of the organisms similarly recovered from 100 cc. of plain broth culture, and resuspended in 10 cc. of supernatant liquid. This additional dosage was occasionally successful, but showed in general little advantage over the smaller amounts. In addition, there was a tendency for weak sera to become entirely inactive under such intensive treatment. Long courses of 10 cc. doses at 4 day intervals were no more successful than the shorter ones. Finally, there was tried a method which had been found satisfactory for many pneumococci.

The animals were given on successive days three doses of 10 cc. each of the whole killed culture. After the lapse of a week followed three more doses, on 3 successive days, each dose consisting of the organisms from 50 cc. of plain broth culture, prepared as before noted. After the lapse of another week, trial bleedings were made, and such animals as were refractory were given a further course of three successive doses, each consisting of the organisms from 100 cc. of broth culture. If at the end of the ensuing week the serum of the animal was still inactive, it was found useless to proceed further. By one or another of these methods, it was found possible to prepare antisera to most of the strains.

4. The technique of the precipitation tests corresponded with that previously described (15), with the exception that in each case 0.1 cc. of the immune serum was used in place of 0.2 cc.

TABLE I.

Strains of Streptococci Employed in This Study.

Source.	Erythrocytes.	Bile.
A4, blood culture; bacterial endocarditis.	Methemoglobin.	Insoluble.
A49, " " rheumatic fever.	"	"
A135, " " " "	"	"
A179, heart valve (necropsy); rheumatic endocarditis.	"	"
2B, blood culture; bacterial endocarditis.	"	"
B3, normal throat.	"	"
B4, " "	"	"
2C, sputum; bronchial asthma.	"	"
C3, " bronchopneumonia.	"	"
2D, blood culture; bacterial endocarditis.	"	"
38D, " " rheumatic fever.	"	"
MB, " " bacterial endocarditis.	Indifferent.	"

5. The complement fixation reactions were performed with the following quantities. 0.1 cc. of bacterial extract was used as antigen. The system was the usual rabbit anti-sheep combination, two units each of complement and of amboceptor being employed. Five descending dilutions of immune serum were used; *viz.*, 0.1 cc., 0.5 cc., 0.25 cc., etc. The antigen-serum-complement mixtures were diluted to 1.5 cc. with saline, and were incubated in the water bath at 37° for 1 hour. At the end of this time, sensitized cells prepared by mixing equal parts of washed 5 per cent sheep cells in saline and of diluted amboceptor were added in amounts of 1 cc. and the mixture was incubated in the water bath at 37° for 45 minutes. At the end of this time the tubes were promptly removed to the ice box, and were allowed to remain there overnight before final reading.

TABLE II.

Complement Fixations with Residue Antigens.

Antigen.	Serum.											
	2B	A49	MB	A179	A135	C3	38D	2C	2D	A4	B3	B4
2B	4	4	2	0	0	0	0	0	0	0	0	0
	4	4	3	0	0	0	0	0	0	0	0	0
	4	4	3	0	0	0	0	0	0	0	0	0
A49	0	4	4	3	3	0	0	0	0	0	0	0
	0	4	4	4	1	0	0	0	0	0	0	0
	0	4	4	4	0	0	0	0	0	0	0	0
MB	0	0	4	4	3	4	4	0	0	0	0	0
	0	0	4	4	3	4	4	0	0	0	0	0
	0	0	4	4	0	4	4	0	0	0	0	0
A179	0	0	4	4	0	4	0	0	0	0	0	0
	0	0	4	4	0	4	0	0	0	0	0	0
	0	0	4	4	0	4	0	0	0	0	0	0
A135	0	0	3	0	4	1	0	0	0	0	0	0
	0	0	4	0	4	0	0	0	0	0	0	0
	0	0	4	0	4	0	0	0	0	0	0	0
C3	0	0	4	4	0	4	4	0	0	0	0	0
	0	0	4	4	0	4	1	0	0	0	0	0
	0	0	4	4	0	4	0	0	0	0	0	0
38D	0	0	0	0	0	1	4	0	0	0	0	0
	0	0	0	0	0	1	4	0	0	0	0	0
	0	0	0	0	0	0	4	0	0	0	0	0
2C	0	0	0	0	0	0	4	0	3	0	0	0
	0	0	0	0	0	0	3	0	4	0	0	0
	0	0	0	0	0	0	1	0	4	0	0	0
2D	0	0	0	0	0	0	2	0	3	0	0	0
	0	0	0	0	0	0	2	0	4	0	0	0
	0	0	0	0	0	0	0	0	3	0	0	0
A4	0	0	0	0	0	0	0	0	0	4	0	0
	0	0	0	0	0	0	0	0	0	4	0	0
	0	0	0	0	0	0	0	0	0	4	0	0

4 indicates complete lack of hemolysis; 3, 25 per cent of cells hemolyzed; 2, 50 per cent of cells hemolyzed; 1, 75 per cent of cells hemolyzed; 0, complete hemolysis.

TABLE II—*Concluded.*

Antigen.	Serum.											
	2B	A49	MB	A179	A135	C3	38D	2C	2D	A4	B3	B4
B3	0	0	0	0	0	0	0	0	0	0	4	4
	0	0	0	0	0	0	0	0	0	0	4	4
	0	0	0	0	0	0	0	0	0	0	1	3
B4	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	0	0	0	0

TABLE III.

Precipitation Reactions with Residue Antigens.

Antigen.	Serum.											
	2B	A49	MB	A179	A135	C3	38D	2C	2D	A4	B3	B4
2B	++	0	0	0	0	0	0	0	0	0	0	0
A49	0	+	+	+++	+	0	0	0	0	0	0	0
MB	0	0	++	++++	+++	+++	0	0	0	0	0	0
A179	0	0	+	++++	0	++++	0	0	0	0	0	0
A135	0	0	+	0	++++	0	0	0	0	0	0	0
C3	0	0	+	++++	0	++++	0	0	0	0	0	0
38D	0	0	0	0	0	0	±	0	0	0	0	0
2C	0	0	0	0	0	0	++	0	+++	0	0	0
2D	0	0	0	0	0	0	++	0	++	0	0	0
A4	0	0	0	0	0	0	0	0	0	+++	0	0
B3	0	0	0	0	0	0	0	0	0	0	++	++
B4	0	0	0	0	0	0	0	0	0	0	0	0

++++ indicates thick, matted precipitate, not broken up even upon violent agitation; +++ indicates same, but less in amount; ++, coarse, flocculent precipitate in bottom of tube; +, definite clouding of liquid, but with little or no settling of the precipitate to the bottom of the tube; ±, faint clouding of liquid, not constantly present in repeated observations; 0, neither clouding nor precipitation.

RESULTS.

These are presented in summary in Tables II and III.

In every instance, a series of controls was made, with normal rabbit serum substituted for the immune serum. In addition, each

of the immune sera was tested by both methods with four antigens prepared as usual from each of four strains of hemolytic streptococci, and also with antigens prepared from a series of pneumococci, including one strain each of Type I, Type II, Type II atypical, and Type III, and ten strains of Type IV. That each of these control antigens was efficient was shown by the production of heavy precipitates when mixed with the corresponding immune horse or rabbit serum. In no case was cross-precipitation or cross-complement fixation noted between non-hemolytic streptococcus sera and either hemolytic streptococcus or pneumococcus antigens. That non-hemolytic antigens will not give cross-precipitation with hemolytic antisera has already been shown (15) and other studies[1] have demonstrated that they are likewise inert when mixed with antipneumococcus sera. The chain of evidence is thus completed, that, as far as concerns these residue antigens, the hemolytic streptococci, the non-hemolytic streptococci, and the pneumococci form antigenically distinct groups.

The entire absence of non-specific flocculations with normal rabbit serum must likewise be emphasized, as contributing further evidence to the specificity of these bacterial extracts.

DISCUSSION.

A glance at the tables shows that the uniformity noted in the hemolytic family is strikingly absent. Some strains are apparently quite isolated individuals; others show a certain amount of cross-reaction between them, but this is not orderly enough to permit it to be used as a basis for classification. It is, however, possible that the study of a larger collection of organisms would display general tendencies which are missing when the number is restricted. No grouping according to source or disease process is evident. Neither is there evidence of the inverse relationship between fixability of antigen and range of antiserum which has been demonstrated by the use of a different technique. The conclusion cannot be avoided that the latter phenomenon, as well as that of cross-reaction between hemolytic and non-hemolytic groups, is not associated with the precipitable substance extracted by the method described. Such cross-reactions

[1] Unpublished data.

may be conceived as being properties of the protein or lipoid-protein fraction of the bacterial cell; or it is possible that the more concentrated antiformin solutions used by Kinsella are able to split the molecule of the precipitable substance into simpler compounds with wider reactive tendencies. Experiments to decide this point are at present under way.

SUMMARY AND CONCLUSIONS.

The applicability to the non-hemolytic streptococci of the technique used for the extraction of a soluble precipitating substance from the hemolytic streptococci is demonstrated.

With this substance as antigen, it is demonstrated by means of precipitation and complement fixation that the non-hemolytic streptococci form an antigenically distinct, but entirely heterogeneous group.

Such cross-reactions as do occur within this group are not useful in the systematic classification of these organisms.

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