THE RELATIONSHIP OF SEROLOGIC GROUPS A, B, AND C OF LANCEFIELD TO THE TYPE OF HEMOLYSIS PRODUCED BY STREPTOCOCCI IN POURED BLOOD AGAR PLATES

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The use of blood agar in the classification of the streptococci was first described by Schottmüller (1903) and more extensively studied by Brown (1919). The latter devised a descriptive system, including hemolytic, partially hemolytic, non-hemolytic, and methemoglobin-forming strains, which is widely used in defining these organisms.

The action of hemolytic streptococci on blood agar has been frequently compared with their cultural and biochemical reactions. However, since the separation of these organisms into groups on a serologic basis by Lancefield (1933), few studies have been made of the relationship between the various serologic groups and the formation of hemolysin.

Todd (1934) and Long and Bliss (1937) have studied the formation of soluble hemolysin by the members of most of these serologic groups and have found them readily capable of forming this substance in every instance. Todd has demonstrated that an antigenic hemolysin is produced only by strains in Group A, and he expressed the opinion that the hemolysin derived from each group possessed demonstrably different reactions to physical and chemical agents. The nature of the type of hemolysis of these varying bacterial products in blood agar was not studied.

Brown (1937; 1939) has recently described the characteristic appearance in poured blood-agar plates of the members of the

serological Group B. Two zones of hemolysis, an inner clear zone and an outer zone containing unhemolyzed red blood cells, were observed under favorable conditions after 48 hours' incubation at 37°C.

It is the purpose of this paper to report the results of a study of 1179 strains of hemolytic streptococci in poured blood agar and to correlate the observed serological and hemolytic characteristics. It will be shown that the type of hemolysis produced by Group A is uniform, characteristic, and distinguishable from all other serologic groups of streptococci, that Group B also has a unique type of hemolysis, and that Group C has a uniform type of hemolysis, although some of the higher groups also produce similar hemolysis. These different types of hemolysis can be observed only when standardized poured blood-agar plates are used and not the more commonly used streak plates.

METHODS

Strains of hemolytic streptococci which had been isolated from patients with various diseases were plucked from streak blood-agar plates for more intensive study. Differentiation of the various strains was made both by means of serologic tests and by the use of pour plates.

Serologic grouping was performed by the micro-precipitin method of Brown (1938), sera for Groups A, B, and C being available.[1]

Poured plates were prepared by inoculating 12 ml. of veal-infusion agar containing 0.7 ml. of defibrinated horse blood with a drop of diluted broth culture, which was then mixed and poured in a $3\frac{1}{2}$ inch sterile petri dish. These plates were observed after 24 and 48 hours in the incubator at 37°, and after 24 hours in the icebox. This is approximately the method previously described by Brown (1919).

The appearance of the surface colonies was studied by streaking a loopful of culture on similar blood agar plates and incubating for 24 hours at 37°C.

[1] We are indebted to Lederle Laboratories, Inc., for supplying us with ample quantities of specific grouping serum.

Hemolysis was recorded as complete when the zone of clearing contained no red blood cells, partial when a large number but not all the cells had been destroyed, and slight if only minimal evidence of hemolysis was present. Plate I shows Groups A, B, and C (magnification $1\frac{1}{2}$ ×). 1) After 24 h. incubation at 37.5°; 2) after 48 h. incubation; 3) after 24 h. refrigeration at 5°.

RESULTS

Group A

Of the 1179 strains studied by the method previously described, 1104 had similar characteristics. All the strains having these characteristics were found to belong to Group A according to the Lancefield classification.

Surface. On the surface of blood agar plates, the colonies were either medium-sized, pearly white, dome-shaped colonies or large, irregular, flat, translucent colonies corresponding to the M and F variants of Ward and Lyons (1935). Zones of complete hemolysis, varying from one to 2 millimeters in diameter, with sharply defined margins, surrounded the colonies of a majority of the strains studied. Around a large number of the colonies, however, there were only small zones of partial hemolysis, even though the same strains in poured plates showed the characteristic hemolysis as described below.

Poured blood-agar plates. At the end of 24 hours' incubation individual colonies were surrounded by an area of almost complete hemolysis and the borders were sharp and clean cut. The hemolytic zones varied from one and one-half to three and one-half millimeters in diameter, the average being two and one-half millimeters.

After incubating the cultures for 48 hours at 37°C., it was noted that the area of hemolysis had increased about one millimeter in diameter and the zone of hemolysis was absolutely clear. No red blood cells could be seen within it when they were examined by the microscope. The margins were very sharp and clean cut. For a given strain, all the zones of hemolysis about a colony were almost exactly the same size. There was only slight varia-

tion, approximately 2 mm., in the size of the zone of hemolysis from one strain to another.

When the plates were again examined after they had been placed in the ice box at 5°C. for 24 hours, the appearance of the colonies and zones of hemolysis showed no change.

From these observations, it seemed clear that serological group A hemolytic streptococci showed the following distinguishing characteristics in blood agar: 1) the zone of hemolysis about the colony was complete; 2) the size of the hemolyzed zone was practically constant for the individual strains; 3) the size of the hemolyzed zones varied very little in different strains compared with the variation in other groups; 4) the border of the zone of hemolysis was sharp and definite.

Group B

Twenty-three strains of Group B were isolated and their features on the surface and in poured blood-agar plates were noted. All strains which were grouped serologically as Group B possessed the following characteristics, and every strain could be placed in Lancefield's serological Group B.

Surface. The colonies on the surface of blood-agar plates were large, flat, and gray in appearance and they were surrounded by a very narrow zone of partial hemolysis. This seemed to be quite characteristic but they were occasionally confused with other serologic groups.

Poured blood agar plates. In contrast to the Group A streptococci, the zone of hemolysis 24 hours after incubation at 37°C. showed an area of incomplete hemolysis about $2\frac{1}{2}$ millimeters in diameter surrounding the colony. The border of the zone of hemolysis was hazy and diffuse, and many incompletely hemolyzed red blood cells were distributed through this zone of incomplete hemolysis.

At the end of 48 hours' incubation the picture had changed somewhat, the zone of hemolysis had increased about one millimeter in size, and it was complete or nearly so. At this time, there was in addition a faint but definite band of hemolysis, about one-half to one millimeter wide, around the clear-cut

hemolyzed zone. When these plates were placed in the ice box for 24 hours, the outside zone of hemolysis had increased in both size and degree until it measured between one and 2 millimeters wide (Plate 1). Therefore, we could conclude that Group B streptococci could be distinguished from the other groups by the band of slight or partial hemolysis surrounding a zone of complete or nearly complete hemolysis. The band occurred in all strains either after 48 hours' incubation or following 24 hours' refrigeration. Brown (1937; 1939) has described the appearance of this distinctive double zoning hemolysis. We have not encountered any Group B strains which failed to show the double zone of hemolysis, although Brown has described such strains.

Group C

There were 19 proved serologic Group C streptococci in the group. They all presented the same type of hemolysis and the same appearance on surface plates, and they could be distinguished quite readily from Groups A and B organisms in poured blood-agar plates. Four more strains which, in poured agar plates, were identical with Group C could not be grouped with serums A, B, or C.

Surface. On the surface the colonies were not entirely distinctive. The zones of hemolysis were larger than those of Group A but they were less complete and the margins were more diffuse.

Poured blood agar plates. In the poured blood-agar plates, after 24 hours' incubation at 37°C., the colonies appeared large, and were surrounded by an area of clear or nearly clear hemolysis which became progressively less so as one approached the periphery. The diameter of the zone of hemolysis was 3 to 5 millimeters but the borders were indefinite so that accurate measurement was impossible. Following 48 hours of incubation, a great increase in the size of the zone of hemolysis occurred, the diameter varying between 5 and 9 millimeters in many instances. Immediately about the colony there was complete hemolysis but the periphery remained very diffuse and was indistinct. Following refrigeration, there was practically no change in the appearance of the colony. In a word, then, the

Group C streptococci could be distinguished easily from Groups A and B by the very large zone of hemolysis which was complete for 3 millimeters or more in the center and which gradually became less complete until it was only slight at the periphery.

Unidentified Strains

Nineteen strains which were isolated did not resemble either A, B, or C and, since those were the only group sera available to us, we were unable to identify them.

Groups D, E, F, G, H, and K

Relatively few strains of Groups D, E, F, G, H, and K were available for study. Those described below were sent to us through the kindness of Dr. James M. Sherman of New York and Dr. E. A. Bliss of Baltimore. Previously they had been grouped according to the serological groups of Lancefield. We wanted to see if these groups had a cultural characteristic in poured blood-agar plates which was distinctive for the group to which they belonged. We were unable to distinguish these groups by this method. Although we had only 2 or 3 strains of each group, there seemed to be no comparison between strains of a given group. However, almost every group contained a strain which was indistinguishable in appearance in blood agar from the appearance of all the Group C strains. None of the strains of Groups D, E, F, G, H, and K were confused with Groups A or B, since they failed to produce either the completely hemolyzed zone with its sharp border, which is characteristic of Group A, or the double zone of hemolysis in Group B.

COMMENT

No satisfactory explanation has been offered for the varying effects of streptococci on blood agar. Evidence is presented in this paper which suggests that at least some of the serologic groups of Lancefield are constant in their various effects on this substance. The underlying mechanisms for these differences remain obscure, except to further suggest, as emphasized by Todd (1934), that the hemolysins of the streptococci of the various groups are distinct substances.

In the 1179 strains discussed in this paper, the type of hemolysis described for the members of Group A has proved characteristic in every instance. Strains of Group B also have always produced a highly distinctive hemolytic pattern. While all strains of Group C were consistent, they could not be distinguished from several strains in the higher groups. From the relatively few strains of the other groups it would not appear that these groups have a characteristic cultural growth in blood agar.

Practically, these variations have proved of great value as checks on serological studies. On various occasions difficulties were encountered in the serologic groupings: one lot of Group A serum showed a false precipitate in all strains; Groups B and C serum required a great deal of care in obtaining the right proportion of serum and antigen and, on occasions, the antigen was not sufficiently strong. On repeated serologic tests they always checked with the blood-agar grouping. Rapid approximations as to the presence or absence of human virulent strains may also be made from material such as throat swabs and sputa if these be suitably diluted and poured in standard horse blood agar plates and examined after 24 and 48 hours' incubation at 37°C. in the absence of available serum.

Brown (1919) has emphasized, and it is important to point out, that these characteristic changes will not be demonstrable unless poured plates are used, as the appearance of colonies on the surface has very little correlation with the widely differing types of hemolysis described in this paper.

From the evidence above, it is likely that the development of hemolysis in blood agar is as constant a function of the organism in certain groups as is the development of group specific substance.

SUMMARY AND CONCLUSIONS

1. Strains of hemolytic streptococci, members of Lancefield's serologic Groups A to K, were studied in poured blood-agar plates.

2. The type of hemolysis produced by strains of Group A was constant and characteristic in every instance.

3. All members of Group B which were studied produced characteristic hemolysis with "double zoning."

4. Members of Group C produced a characteristic hemolysis

which, however, could not be differentiated from various strains in higher groups.

5. The few strains of Groups D, E, F, G, H, and K did not show a group-specific hemolytic effect in blood agar.

6. It is suggested that the appearance of streptococci in poured blood-agar plates may be used as a check on serologic tests and as a satisfactory method of approximating the group of unknown strains in the absence of grouping serum.

7. Standardized blood-agar plates must be used or the described changes will not be observed.

We wish to thank Miss Eleanor Fleming for her assistance in the serologic grouping.

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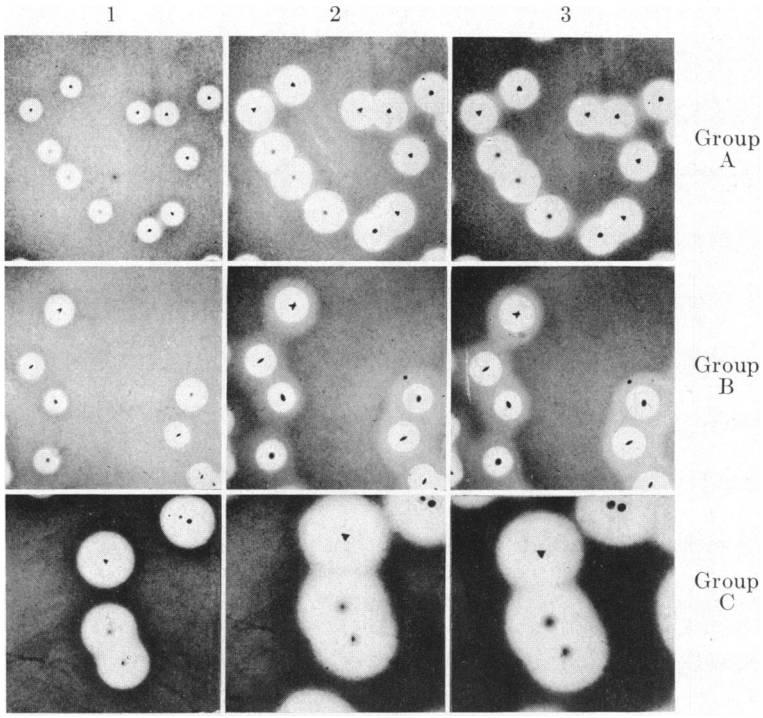
PLATE 1. HEMOLYSIS AFTER: (1) 24 H. AT 37.5°; (2) 48 H. AT 37.5°; (3) AN ADDITIONAL 24 H. AT 5°



(Lowell A. Rantz and Marjorie L. Jewell: Serologic Groups and Hemolysis)