THE IDENTIFICATION OF THE HÆMOLYTIC STREPTOCOCCI, WITH SPECIAL REFERENCE TO THOSE OF THE UPPER RESPIRATORY TRACT

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SINCE the streptococcus was first suspected as being the ætiological agent in acute rheumatism evidence has been gradually accumulated in support of this statement, and, since Coburn (1931) named the hæmolytic streptococcus as the causative variety, much additional work has been done. At the 1938 meeting of the British Medical Association many speakers supported this view, Green (1938) reporting a series of 200 cases of acute or subacute cases of rheumatism (58 per cent. had hæmolytic streptococci in the naso-pharynx at the onset of the rheumatic syndrome), and Gordon (1938) reported experimental work which appeared to reconcile the streptococcus and virus theories of the ætiology. In this work arthritis was produced in rabbits by a combination of vaccinia virus and hæmolytic streptococci.

In view of the importance of the hæmolytic streptococci, most probably as the actual cause, or as an essential contributory factor, the accurate identification of the hæmolytic streptococcus must be of great importance. Willcox (1938) stressed the importance of recent advances in bacteriological technique as a cause of the advance in the knowledge of the ætiology of acute rheumatism.

In this communication methods of isolation, appearance on blood plates, hæmolysin production, fibrinolytic activity, colony appearance, and serological methods will be discussed. It will be noted that discussion of fermentative reactions has been omitted. The variability of such reactions in the case of the hæmolytic streptococcus and the difficulty of reconciling the grouping by this method with other classifications seem sufficient reason for the exclusion of the fermentative reactions as a means of differentiation.

METHODS OF ISOLATION FROM THE UPPER RESPIRATORY TRACT

(1) *From the Throat*.—Throat swabs were taken in a manner similar to that used in the examination for *C. diphtheriæ*. The swab was then applied to an agar plate containing 5 per cent. rabbit blood. The material from the swab was deposited by lightly touching the plate and then spread over the plate by a wire spreader. The plate was then incubated at 37° C. overnight and examined for colonies showing a clear ring of hæmolysis. When such colonies appeared they were picked off and inoculated into semi-solid agar for further investigation. The choice of rabbit blood for plates and the reason for the further test by the extended method will be discussed in a later section.

(2) *From the Naso-pharynx*.—West's swabs were used and specimens obtained in the usual manner. Plates were inoculated and examined as in the case of throat swabs.

(3) *From Enucleated Tonsils*.—Tonsils received after tonsillectomy operations were treated in the following manner: Both tonsils were macerated in 5 c.c. of nutrient broth, and filtered through a double layer of sterile gauze. A loopful of the filtrate was then inoculated on to a rabbit blood agar plate and examined in the usual manner. Repeated inoculations of the filtrate were made if the initial inoculation of the filtrate proved unsuccessful. When the streptococci were overgrown by staphylococci the technique of Haxthausen (1927) was used, crystal violet 1/40,000 and 1/80,000 being employed to restrain the staphylococci in broth culture. Blood plates were inoculated from the broth cultures after overnight incubation. In this way small numbers of streptococci were able to grow sufficiently in the broth to produce a considerable number of colonies on the plate.

APPEARANCE ON BLOOD PLATES

Schottmüller's (1903) use of the blood plate to differentiate hæmolytic from non-hæmolytic streptococci was accepted by most as the method of choice, although McLeod (1912) and Lyall (1914), investigating hæmolysin production by tube methods, found that the blood agar plate method, if unsupported by other tests, was unreliable. An improved technique in the blood plate

method was introduced by Brown (1919). Later Cumming (1927) further emphasised the unreliability of the blood agar plate and demonstrated that certain strains appearing hæmolytic on human blood agar plates were not hæmolytic on rabbit blood agar or by tube tests. These strains he designated pseudo-hæmolytic. Despite the repeated demonstration of the fallacy of the unsupported blood agar plate test investigators relied on this method for many years. In recent years, however, an increasing use has been made of the tube test to corroborate plate findings. Cumming (1927) has suggested that this failure to realise the incompleteness of the blood plate method has led many observers to include non-hæmolytic strains in a hæmolytic series. The non-hæmolytic strains are notoriously difficult to classify, and, introduced into a group of hæmolytic strains, could cause endless confusion.

The standard plates of Brown (1919) appear to have all the ingredients standard except the type of blood used. Horse blood appeared to have been used, but the limits laid down for standard plates do not preclude the use of blood from other animals. In view of Cumming's work the type of blood used should also be standard in plate work.

In an investigation of eighty-three consecutive specimens of sputum from tuberculous patients, sixty specimens were found to have streptococci which gave clear rings of hæmolysis on 5 per cent. human blood agar plates. Of the sixty strains thus isolated, only four gave similar clear zones on 5 per cent. rabbit blood agar. The sixty strains thus isolated were replated, using blood plates containing human, sheep, horse and rabbit blood. The 5 per cent. human blood plates were used as a standard and by colorimetric methods the other plates adjusted to a uniform colour. Incubation of the plates resulted in the following findings: Of the sixty strains which gave hæmolytic rings on human blood plates, fifty-three gave hæmolytic rings on horse blood plates, forty-seven on sheep blood plates, and four on rabbit blood plates. Thus hæmolytic rings were most readily obtained when human blood plates were used and slightly less readily in the case of horse and sheep blood plates. Rabbit blood plates gave the fewest strains showing hæmolytic rings. In subsequent tests, as described in the next section, the four strains which were hæmolytic on rabbit blood plates were the only strains to produce hæmolysin in the tube test. The experiments showed that

division of streptococci of a certain character into hæmolytic and non-hæmolytic types by means of the blood plate method depended to some considerable extent on the type of blood used. Human, sheep and horse blood were all more readily lysed by certain strains on an agar plate than was rabbit blood. The streptococci mentioned were isolated from sputum and can be considered as a possible source of error if not identified as pseudo-hæmolytic in a search for hæmolytic strains.

HÆMOLYSIN PRODUCTION AS SHOWN BY THE TUBE TEST

McLeod (1912) improved the then existing technique for the production of hæmolysin. He grew the organisms for twenty to twenty-four hours and then added 1 c.c. of a 5 per cent. suspension of washed ox blood cells. The smallest quantity of culture causing hæmolysis was the minimum hæmolytic dose. The culture medium used was peptone broth, with a small percentage of serum added.

In the following experiments the technique of McLeod was closely followed. Whole cultures were used in preference to filtrates. Serial dilutions of eighteen-hour cultures were made and an equal quantity of a 3 per cent. suspension of washed blood cells added, the final volume being 0.5 c.c. The tubes were incubated at 37° C. for ninety minutes. Each strain tested was diluted 1 in 2, 1 in 4, 1 in 8, 1 in 16, 1 in 32, 1 in 64 and 1 in 128 for the test; saline and serum controls were used. The optimum percentage of serum in the serum broth for the production of hæmolysin varied with the type of serum used. With human serum 50 to 60 per cent. gave the greatest production of hæmolysin, 50 per cent. was the optimum figure with horse serum, 60 per cent. with sheep serum, and 70 to 80 per cent. with rabbit serum. It should be noted that the term "rabbit serum" is used here denoting tame rabbit serum, tame rabbits being used exclusively. This point has been raised, as Cumming found that with wild rabbit serum 30 per cent. was the optimum figure for hæmolysin production. Investigation of the time factor showed that eighteen hours was the optimum time; this time had been fixed by McLeod as his standard. Delay in the examination of cultures may lead to errors, as hæmolysin is very labile, there being rapid deterioration after twenty-four hours with whole cultures. In the main, whole cultures show hæmolysin pro-

duction over a more extended period than do supernatant fluids. There was some evidence, in limiting experiments, to show that the time of maximum hæmolysin production varied with the concentration of serum in the culture medium; as the percentage of serum used in the tests was that which was best suited to the production of hæmolysin when the incubation time was eighteen hours, there was little to be gained by following this line of investigation. It was also found to be important to use the appropriate cells when testing a serum broth culture—*e.g.*, when using horse serum to use horse cells, and similarly with other types. Some strains, actively hæmolytic when tested with corresponding serum and cells, did not give lysis when the cells and serum in the test were not of the same kind. Several experiments were conducted in which the hæmolytic streptococcus was grown in serum broth containing the serum of one species, and after eighteen hours' incubation these cultures were tested for the presence of hæmolysin by cells from another species. In all, twenty-eight strains, actively hæmolytic on blood agar plates and by McLeod's definitive test, were tested by three methods simultaneously, using eighteen-hour cultures. These three methods were:

(1) 70 per cent. rabbit serum broth, using 3 per cent. washed rabbit cells.

(2) 50 per cent. horse serum broth, using 3 per cent. washed sheep cells.

(3) 60 per cent. sheep serum broth, using 3 per cent. washed sheep cells.

Of the twenty-eight strains tested, all were hæmolytic by methods (1) and (3)—*i.e.*, by McLeod's method. In method (2), however, only eighteen strains showed evidence of hæmolysis; the other ten strains showed no sign of hæmolysin production.

This type of experiment could be extended to include numerous varieties of serum and cells, but the above example should suffice to show that fallacious results may be obtained when the serum and the cells are of different kinds.

The sixty strains isolated from sputum, as described in a previous section, were investigated by the definitive method, and only the four strains which gave rings of hæmolysis on rabbit blood agar gave hæmolysis in the tube test. It was thus shown that rabbit blood agar was essential in the plate test for showing true hæmolysis, as it was found in the tube test. Although

not found to be essential in the tube test, rabbit serum and cells were used throughout, giving uniformity with the blood plates. The pseudo-hæmolytic strains failed to show hæmolysin by tube tests. The ring of clearing in human, horse and sheep blood agar plates, in the case of the pseudo-hæmolytic strains, is evidently not caused by hæmolysin. In view of the above findings it would appear that the term "hæmolytic" as applied to streptococci should be used with caution.

The work of McLeod, Lyall and Cumming, although now generally recognised as significant, still receives in some quarters less attention than one would expect. Many still rely on the blood plate alone, very often blood plates of horse or human blood, which, as shown above, are excellent for the demonstration of pseudo-hæmolytic strains.

FIBRINOLYTIC ACTIVITY

The original work of Fry (1910) on the fibrinolytic activity of hæmolytic streptococci was elaborated by Tillet and Garner (1933, 1934). The method employed to detect fibrinolytic activity is to clot human plasma, by the addition of calcium chloride, in the presence of a serum broth culture of the organism to be investigated. If the organism be fibrinolytic the clot formed will be lysed. Conversely, human plasma can be tested against a known fibrinolytic strain. This converse experiment can be applied to the investigation of rheumatism. Hadfield, Magee and Perry (1934) used this method in investigating acute rheumatism, as did Stuart Harris (1935) in cases of rheumatoid arthritis.

The technique has been modified repeatedly, the method of Colebrook now being generally adopted. In view of the fact that antifibrinolytic power has been demonstrated in the plasma used by the present writer, a method was devised, founded on Colebrook's, in which pooled plasma was used. The preparation of the pooled plasma and the method of performing the test has been previously described (Stewart, 1936).

Of the streptococci tested, only the hæmolytic variety lysed the clot formed. Thirty-eight strains from scarlet fever cases gave lysis and seven did not. Of thirty-two hæmolytic strains from tonsils, only two did not show lysis. None of the non-hæmolytic or pseudo-hæmolytic strains lysed. Thus 88 per cent.

of the hæmolytic series were fibrinolytic. In a larger series tested (269 strains) the findings were similar. Reviewing the findings, it was pointed out that probably about 10 per cent. of patients suffering from infection with hæmolytic streptococci have been infected with non-lysing strains (Stewart, 1936). This would appear to place limitations on the use of the above method as evidence of previous infection with the hæmolytic streptococcus.

The testing of plasma of young patients for antifibrinolytic power as evidence of a previous attack of acute rheumatism, either detected or undetected, has been encouraging from the point of view of demonstrating the ætiological significance of the hæmolytic streptococcus. The same may be said of the work in connection with plasma from the middle-aged in the study of rheumatoid arthritis. It should be pointed out that antifibrinolytic power may be developed in response to other infections. Tillet and Garner record antifibrinolytic power following various streptococcal infections. The writer's own blood is strongly antifibrinolytic, and this is taken to be due possibly to some non-rheumatic infection. The widespread nature of hæmolytic streptococcal infection has made it difficult to assess the value of evidence of such infection as denoting the hæmolytic streptococcus as the ætiological agent in any particular disease. At present there would appear to be insufficient evidence of the antifibrinolytic power of a sufficient number of controls to allow of the true significance of the results in pathological cases being appreciated. There is no record of work to show whether a hæmolytic streptococcus, which does not lyse fibrin *in vitro*, can produce an antifibrinolytic response in a person infected by that organism.

In spite of these possible limitations of the antifibrinolytic test, the work on acute rheumatism and rheumatoid arthritis by this method has been very encouraging.

COLONY APPEARANCE

Cowan (1922) described rough and smooth colonies of streptococci and Eagles (1924) also described two types of colonies. Griffith (1927) described three types: (a) opaque, flat, rough colony; (b) colony with raised opaque centre; (c) mucoid, large colony. Walker (1923), quoted by McLeod (1929), noted mucoid colonies of hæmolytic streptococci which proved more virulent

than the colonies present on the plate. Remarking on this, McLeod said: "If it is possible to establish a relationship between the varied morphological appearance of colonies and pathogenic significance much time and effort may be saved."

Todd has done much detailed work on colony appearance, and the maintenance of virulence in culture by the addition of a catalase to prevent the accumulation of hydrogen peroxide. The reader is referred to the publications of Todd (1927-1930) on this subject.

In a series of experiments conducted on similar lines, it was found that variation in the type of blood and in the type of peptone used gave considerable variation in the type of colony obtained. Murray (1935) demonstrated that the colony appearance of *C. diphtheriae* was influenced by the drying of the plates. In the same way it was shown in the present work that the state of the plate when inoculated influenced the colony appearance of the hæmolytic strains, as did variation of the strength of the agar. Plates were inoculated in duplicate and striking differences obtained. In addition, it was also observed that if there was any crowding of the colonies, the "smooth" type of colony was more in evidence, although the same strain with widely separated colonies showed "rough" colonies. Although moisture and dryness, strength of agar, and method of examination all tended to give different results, and although no change in colony type was observed after the aeration of cultures as described by Todd, there was more evidence in favour of Todd's assertion that smooth colonies were a degenerate type as opposed to Cowan's statement that the rough colonies were degenerate. It was observed that smooth colonies gave poor growth and tended to die out. In many instances an apparent luxurious growth of smooth colonies proved, on closer examination, that the smooth nature of the colonies was due to overcrowding and that replating with a thinner spread showed the colonies to be of a different character.

SEROLOGY

The classification by serological methods has been frequently attempted. A variety of methods has been employed, the absorption of agglutinins method and the precipitin method being the most recent. The technique adopted by Gordon and his co-workers (1917) in the serological classification of the

meningococcus and by Tulloch (1922) working with the gonococcus was adopted by Cumming (1927) with success. No uniform technique has been used by the various investigators working with streptococci, and the writer decided to use a technique which proved acceptable in work with other cocci.

Difficulty in obtaining adequate suspensions has been frequently observed. Andrewes (1932) obtained only 48 per cent. of his strains in adequate suspension. Cumming (1927) dried and ground his strains to obtain satisfactory suspensions. This was the method previously used by Tulloch and his co-workers (1924) in their work on the serological classification of the tubercle bacillus. The drying and grinding method gave consistently satisfactory suspensions; it is undoubtedly a laborious process, but no satisfactory substitute was found. The preparation of antisera, the method of absorption and subsequent agglutination tests with suitable controls have been fully described (Cumming, 1927). Of 157 strains from surgical sources, 136 absorbed our stock antiserum—*i.e.*, 87 per cent. of the surgical strains were of one type serologically. Gordon (1922) showed that 90 per cent. of his surgical hæmolytic strains were of one group. Of Griffith's twenty-eight types, twenty-two completely absorbed our stock antiserum, the remaining six giving either incomplete or no absorption.

In Lancefield's (1928) precipitin method, strains have been classified according to a group specific substance; in all, seven groups have been described. Group A contains the majority of the strains pathogenic to man and would appear to correspond to the large group previously described by Gordon (1922) and others. Lancefield has also described a type specific substance protein in nature. Griffith has obtained twenty-eight different types in Group A, using a slide agglutination method. He also uses absorbed sera for the slide tests. Andrewes (1932) commented that this method "is lacking in refinement and is unadapted to exact quantitative methods." Perhaps the statement of Stevens and Dochez (1926), who, after studying their strains by absorption of agglutinins, stated that they had found "that the antigenic individuality of each strain is a more marked feature than any group characteristic," throws light on the difficulty of choice of method and of interpretation. From one aspect, the hæmolytic streptococci contain one large group of about 90 per cent. serologically identical strains, from another aspect

there seem to be innumerable subdivisions. After seven years' study Andrewes (1932) came to the conclusion that "the more one studies hæmolytic streptococci, the more strongly is the impression gained that they are in a state of constant flux in which it is difficult to find any firm foundation for a permanent systematic classification."

As has already been noted, it would appear to be entirely a matter of opinion whether the group specific character is accepted as a means of classification or whether further subdivision into numerous types is required to satisfy the reader.

Eagerness to classify the hæmolytic streptococci in the hope that an effective therapeutic serum would be produced gave an impetus to this work in the past. The effective actions of the sulphonamide group of drugs has probably removed this incentive—for the present, at least. Without some such incentive there would be a tendency for this somewhat disheartening work to be neglected. The recent work on acute rheumatism, however, has maintained the importance of the serological classification in the study of epidemiology, especially of strains from the nasopharynx, and should produce an additional stimulus to such work. Too much stress cannot be laid on the necessity of the employment of standard methods in all investigations if we are to profit by the correlation of results.

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