TYPES OF HAEMOLYTIC STREPTOCOCCI IN RELATION TO SCARLET FEVER (SECOND REPORT).

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IN my first report¹ on the classification of haemolytic streptococci I have stated that 45 per cent. of a series of 81 scarlatinal strains fell into three serological types, the remainder being made up of apparently heterogeneous strains.

Application of tests with agglutinating sera, prepared with the three scarlatinal types, to 46 cultures of haemolytic streptococci from puerperal fever revealed a distinction between the two series of strains. In the puerperal series heterogeneous strains were in a greater majority than in the scarlatinal, and, while two of the scarlatinal types were represented, the third, Type II, was not found.

Though the existence of a single serological type of streptococcus, occurring only in association with scarlet fever and not in other diseases, would have been a stronger argument in favour of the streptococcal origin of scarlet fever the above observations, nevertheless, unquestionably support that view. One would anticipate as appears to be the case, that the causal organism would more often show identity of type in the epidemic disorder than in the sporadic puerperal fever.

In view of the desirability from the epidemiological standpoint of establishing the incidence of the serological types of streptococci in scarlet fever and of developing a reliable method of identification, I have undertaken this investigation of a further and larger series of strains of haemolytic streptococci from scarlatinal sources.

I wish to express my great indebtedness to the Medical Superintendents of three of the Metropolitan Fever Hospitals, Dr Alexander Joe, Dr J. Wilkins, and Dr T. H. Woodfield, who supplied me with swabs from the throats of scarlet fever cases and clinical notes on the subsequent course of the disease. I have also to thank Dr W. Gunn for allowing me to use the results of his analysis of the clinical data in those cases from the Park Hospital from which I obtained swabs.

MATERIAL AND METHODS.

The cultures of haemolytic streptococci were obtained from throat swabs which had been taken for me by the Medical Officers at the Park, Eastern and North-Western Hospitals of the Metropolitan Asylums Board during six

¹ Griffith, F. (1926). Types of Haemolytic Streptococci in Relation to Scarlet Fever. *Journ. Hygiene*, 25, 385-397.

weeks in October and November, 1926. The cases of scarlet fever were swabbed as early as possible after admittance to the hospital.

On being received at this laboratory the swabs were plated on fresh blood agar and were examined after incubation overnight. Many of the plates yielded large numbers of haemolytic colonies, often practically in pure culture. At first five colonies were picked off and subcultivated, but after a time the number was reduced to three, as in no case had any differences in the reactions of individual colonies been detected. Each colony was grown overnight in broth plus rabbit blood and the tubes were stored in the refrigerator. These stock cultures remain viable for a long period and subcultures can be made from them when required.

Agglutination tests were made upon each strain as soon as possible after isolation and generally in the second subculture. All the strains were tested against the three Type sera, I, II, III, by the method of agglutination described in my first report, viz. a drop of 1 in 20 dilution of serum is mixed on a slide with a drop of a fairly strong suspension, the process being watched under a low power dissecting microscope.

It was found that the deposit from serum broth (5 per cent. bovine serum in trypsinised meat broth of pH 7.6) culture made a suitable suspension for this test, the presence of the serum not interfering with the reaction. The growth, although it had generally fallen to the bottom of the tube, leaving the supernatant broth clear, could be worked up into a more finely granular suspension than that in plain broth; only a few strains produced both in plain broth and in serum broth a culture which was invariably too coarsely granular for use.

It has happened frequently that streptococci which have remained in suspension during growth in broth have after centrifuging formed a more coarsely granular deposit than those which grew at the bottom from the outset.

After the preliminary sorting with Types I, II and III sera, a number of strains were taken for the preparation of agglutinating sera in rabbits. Some of these strains had already given positive agglutination reactions with one or other of the above three sera, viz., five of Type III and one of Type I; they were chosen with the object of testing whether their agglutinogenic qualities would confirm their classification by agglutination. In addition, several strains which had failed to agglutinate were used for serum preparation, and amongst these were found two identical strains which subsequent analysis of the whole series showed to be of not uncommon occurrence, and to represent therefore a fresh type. This has been designated Type IV.

Agglutinin absorption experiments, carried out as in my first investigation, were made on a number of strains; they confirmed entirely the results obtained by the direct method of agglutination. I originally defined the types by means of absorption, but this test with streptococci, which do not produce an abundant growth on solid media, is very laborious and hardly practicable

as a routine on any considerable scale. The slide method of agglutination which avoids the difficulty of obtaining suspensions sufficiently stable for incubation in the water-bath is simple and economical, and large numbers of strains can be quickly tested against a variety of sera.

The result of agglutinin absorption may also be tested by this slide method. This modified absorption method is as follows: the deposit of 100 c.c. of broth culture contained in 1.25 c.c. of the medium is added to 0.1 c.c. or 0.05 c.c. of the serum to be absorbed; the absorbing culture, especially if inclined to be granular, is thoroughly mixed with the serum by means of a teat pipette, the point of which is pressed against the bottom of the tube. Since the union of agglutinin and antigen takes place immediately they are brought into contact with each other, it is unnecessary to allow the mixture to stand or to incubate in the water-bath with possible weakening of the agglutinating power of the serum; the mixture may be centrifuged immediately and the tests for absorption of agglutinin made as soon as the clear supernatant fluid is obtained.

The tests for the presence or absence of agglutinin in the treated serum are made as in the direct agglutination tests; a large drop of serum dilution is mixed on a slide with a small drop of a thick suspension of the strain homologous to the serum, equal density being aimed at in different tests. On the same slide are placed, as controls, drops of the suspension mixed (1) with broth, (2) with untreated serum.

RESULTS OF SEROLOGICAL ANALYSIS.

Table I shows the results of tests with agglutinating sera, prepared with four types of scarlatinal streptococci, upon 222 strains of haemolytic streptococci obtained from the throat swabs of scarlet fever patients in three Metropolitan Fever Hospitals during the period referred to.

Table I.

Hospital	No. of strains	Classified with streptococcal sera				Unclassified
		Type I	Type II	Type III	Type IV	
Park	68	2	27	12	16	11
Eastern	84	2	19	16	17	30
N. Western	70	6	11	17	11	25
Totals	222	10	57	45	44	66
Percentage	—	4.5	25.6	20.3	19.8	29.7

The number of strains which could be classified under one or other of the four types is 156 out of a total of 222 examined = 70.2 per cent.

The proportion of classified strains is thus much higher than in my first series (45 per cent.). This is due to the inclusion of a fresh type, Type IV, which occurs as frequently as does Type III, but which had not been defined at the time of the previous investigation. There is also to be noted a smaller proportion of Type I strains in this London series compared with the first

series which contained strains from cases of scarlatina in London, Manchester and Liverpool. It is a curious fact that most of the ten Type I strains in this second series produced suspensions which were too coarsely granular for decisive test by direct agglutination; their type was suspected since on the addition of Type I serum, the granules became much coarser, but agglutinin absorption tests were necessary to establish their identity with Type I.

Practically all the Type II strains gave fine stable suspensions which immediately formed coarse clumps on the addition of the type serum. In three instances the Type II colonies on the direct plates from the throat could be emulsified as readily as pneumococcus colonies and it was possible to use the actual mass of the colony for agglutination test. Type IV strains were also well defined. With strains of Type III, it was occasionally necessary on account of granular suspensions to make repeated tests, but when a satisfactory suspension was obtained the type was as well defined as the other three.

The majority of the types of streptococci in this series have been identified by agglutination tests only, but the results have been confirmed in many instances by absorption of agglutinin, especially where, owing to granular or auto-agglutinating suspensions, there has been any doubt as to the validity of the direct observations.

The 66 unclassified strains, excepting five with which serological tests were found to be impossible owing to their very coarsely granular growth, were alike in their failure to agglutinate with the four chief type sera. In order to classify this group of strains, it will be necessary to prepare from them a considerable number of agglutinating sera, as, according to some preliminary tests, there are many individualistic strains.

On the other hand, sera prepared from certain of these unclassified strains appear to have a wide range of action and in this respect they resemble sera from strains in the puerperal series of haemolytic streptococci. For example, one of these sera from a scarlatinal strain agglutinates a large number of strains which are also picked out by a puerperal streptococcus serum; in addition the former serum, although the strain producing it is not affected by Type I serum, produces clumps with one Type I strain, but not with another. The bearing of these results on the serological classification is discussed later.

CHARACTERS OF HAEMOLYTIC STREPTOCOCCUS COLONIES ON BLOOD AGAR PLATES.

The medium on which the colonies were grown was composed of nutrient agar made with trypsinised meat broth together with 3 to 5 per cent. of fresh oxalated horse blood. The mixture was poured into plates in which a thin layer of agar without blood had been allowed to set, as recommended by Gordon. Haemolytic colonies produced on this medium a sharply defined clear zone of varying width; they were readily recognised even when they were sparsely distributed on a plate so closely covered with other bacterial

colonies that it was impossible to obtain a pure culture without a second plating.

The following description of the different varieties of haemolytic streptococcus colonies has been made from plate cultures of pure strains. The same strain has been plated several times and the colonies kept under observation for three or four days in order to note the changes which take place as the colonies age and dry. As a rule the typical characteristics have developed in an isolated colony after two days' incubation.

Although streptococcus colonies are at first sight so diverse in appearance, most of the varieties can be identified with one or other of the following:

(1) The first, which is of most frequent occurrence, consists of an opaque flat or slightly convex disc with a more or less rough surface; it is coherent and can be picked up as a whole with a platinum spatula, on which it curls up in the shape of a saucer, leaving a pit in the medium. As the medium dries, the edges of the colony become wrinkled and detached from the surface until the colony finally rolls up like a dried leaf. This is the appearance of a well grown colony which has had plenty of room to grow and the chief characteristic is the tough consistency of the growth. In its early stages such a colony may be small, rounded and soft in consistency, gradually becoming more opaque and porridgy. In the centre of the plate and where the colonies are crowded they may cease growth at various stages of their development, and in this condition may be difficult to distinguish from the next variety.

(2) The second variety of colony, as indicated above, resembles the first in the early stages of growth; its distinguishing characteristic is that the soft consistency of the growth is retained; these colonies do not develop into the coherent discs which can be lifted from the medium as a whole. A well grown colony shows a soft whitish opaque raised centre, often cone-shaped, and a thinner translucent margin.

(3) The third variety of colony may appear in cultures which ordinarily produce colonies of one or other of the first two described above. The conditions which cause this form to appear are obscure, but it is certain that a moist medium is necessary. A fully developed colony of this third variety, one which has had ample medium on which to grow, consists of a shiny transparent dome, almost watery or slightly mucinous in consistency, thus resembling a Type III pneumococcus. This clear colony may develop from an opaque disc of 24 hours' incubation if the plate, after removal from the incubator is allowed to remain on the bench at room temperature; a few whitish striae may occasionally be seen in the centre and be all that remains of the original colony.

If a watery colony is plated, the colonies produced may be of the original opaque character or may be flat and translucent with a contoured surface, some of which may again develop into the clear dome-shaped type. The transformation is apparently mucoid in nature but does not affect the staining affinities of the majority, at any rate, of the bacteria themselves, since smear preparations show abundant well-staining cocci. One may surmise that the individual bacterial elements become separated by a thin mucoid secretion, thus rendering the whole colony transparent. All the serological types of scarlatinal streptococci as well as strains of puerperal origin may produce cultures consisting partly or wholly of colonies of this third variety.

On the other hand, with varieties 1 and 2 there is to some extent correlation with serological type; for instance, one can generally recognise a Type III culture from its appearance on blood agar plates. Type III scarlatinal streptococcus almost invariably produces colonies wholly of the second variety,

the chief characteristic of which is that they remain soft and cannot be picked off as a whole even after several days' incubation. Type I cultures are either wholly composed of colonies of the first variety or show a mixture with colonies intermediate between the first and second.

In strains of other serological types the tough consistent colony of the first variety is of most frequent occurrence.

I have given some attention to the possibility that there may be obtained from streptococcal cultures rough and smooth colonies analogous in their differential characters to those obtained from pneumococcal cultures, but I have searched in vain for them.

At first I entertained the idea that the dome-shaped watery colonies of the third variety might be the equivalent of the smooth virulent pneumococcus colony, especially as they occurred frequently on blood agar plates sown with the blood of a mouse which had died of a streptococcus infection. However, subcultures made from the coherent or watery colonies of the same strain did not show differences in virulence and reacted equally well with the appropriate agglutinating serum prepared against the whole strain. It is true that the actual watery colonies from the plates, when suspended in broth and tested with agglutinating serum, produced only very small clumps, but the same colonies grown either in broth or on plain agar slants gave the characteristic reaction of their type. The defective agglutination of the suspensions of the watery colonies must, I somewhat reluctantly concluded, be attributed to the physical characters of such suspensions and not to differences in antigenic structure between them and the ordinary coherent colonies.

Apart from the colonies of the third variety, I have subcultured in broth from a plate culture numerous colonies showing slight differences in appearance but have invariably failed to find any distinction between them in regard to their agglutination reactions with the serum of the type to which they belong.

Passage from mouse to mouse for many generations has not induced any constant alteration in the colony characteristics nor has any effect of that nature been produced by growth in streptococcus immune serum.

There is no consistent relationship between variety of colony and the character of the suspension when subcultured in broth. Both coherent and watery colonies of streptococci may produce at one time uniform growths and at another granular growths which deposit at the bottom of the broth.

Mary Cowan¹ has shown that a culture of haemolytic streptococcus will yield strains of different grades of virulence for mice; the more virulent strains grow with even turbidity in broth while the less virulent form a precipitate; the former she has called smooth, the latter rough. As she has not determined whether the two varieties are capable of being differentiated serologically, it must remain an open question how far the streptococcal

¹ Cowan, Mary L. (1922-1924). Variation Phenomena in Streptococci. *Brit. J. Exper. Pathol.* 3, 187; 4, 241 and 5, 226.

varieties she describes correspond to rough (avirulent) and smooth (virulent) pneumococci. It may, however, be pointed out in opposition to Cowan's conclusions that the streptococcus of Type II, grown directly from the scarlatinal throat and presumably in a virulent state, generally forms a very coherent colony which nevertheless produces a uniform stable suspension when grown in broth. Such stable cultures in turn yield only coherent colonies when plated out on blood agar.

Serological Types of Streptococci from two or more members of the same family attacked by Scarlet Fever.

Table II shows the number of instances in this series of swab cultures where the same type of haemolytic streptococcus was obtained from two or more scarlatinal cases in the same family.

Table II.

Type	No. of instances	No. of infected members of family
II	4	2 in each
III	2	2 in each
Unclassified strains	3	4 in one, 2 in the rest

In one instance a very granular Type I strain was obtained from one child, while from the brother a strain grew which could not be classified with the four type sera. The following occurrence may also be mentioned. Two children from the same family were sent into hospital, one having developed an apparently typical scarlatinal rash, while scarlet fever was suspected in the other though no rash had developed. Bacteriologically they were alike, since in neither case were haemolytic colonies obtained from the throat swabs.

Clinical form of scarlatina in relation to serological type of Haemolytic Streptococcus.

The clinical data were collected by Dr W. Gunn at Park Hospital in 100 of the cases which I examined bacteriologically, and I am indebted to him for permission to quote from his unpublished report. These comparisons of the serological and clinical observations, which were made quite independently, show that the more severe forms of scarlet fever, including the single fatality, were found in cases from which Type II was obtained. Type IV apparently caused the mildest form of disease; in one case after an initial infection with this type a relapse occurred, but the second attack was unfortunately not investigated bacteriologically.

There were not sufficient numbers of Type I strains in this series of 100 from which to form a conclusion upon the severity of disease produced by this type.

A few instances may be given to show that cultures from the throat, especially if combined with a test of the type of haemolytic streptococcus, may be of interest to the clinician in doubtful cases of scarlet fever.

Case 1 gave considerable difficulty in diagnosis; during the course of the illness, which was not severe, it was considered to be scarlatina with an atypical rash. The Dick test was positive 18 days after admission and as desquamation was not typical the patient was discharged as a case of erythema.

The throat swab yielded on two occasions numerous haemolytic streptococci which were of the mild scarlatinal Type IV.

Case 2 was doubtful and, as there was no desquamation during a period of 73 days from the beginning of the illness, was decided finally not to be scarlatina. Nevertheless the throat swab yielded haemolytic streptococci which were of the unclassified group.

Case 3 had a typical rash and profuse desquamation. On admission there was a prepatellar abscess from which pus was evacuated, yielding haemolytic streptococci.

No haemolytic streptococci were found in the throat swab. In this case the inflamed bursa was apparently the primary focus and the site of production of the specific toxin.

Case 4 from another hospital was admitted as scarlet fever, but on the 21st day of illness had a so-called relapse. The throat swab on admission was negative and the clinician came to the conclusion that the primary infection might not have been scarlatinal.

Seven cases from the Park Hospital yielded no haemolytic streptococci from the throat swab. On consulting the clinical data it was found that five of these cases, which on admission had been regarded as doubtful scarlet fever and had been put in observation wards, had been discharged (without reference to the result of examination of the swabs) as not having been cases of scarlet fever; two of them gave previous histories of scarlatina and, on admission, gave negative Dick reactions, while three were Dick-positive throughout the period of observation. The remaining two cases with negative swabs were regarded as clinical scarlet fever; they showed definite desquamation and became Dick-negative reactors. It may be conjectured that in these two cases there was either a failure of technique or a different site of infection and toxin production than the throat.

DISCUSSION.

This study of a series of haemolytic streptococci from scarlatinal sources reveals a differentiation into serological types closely resembling that found in the pneumococcal and meningococcal groups. The streptococcal types are less sharply defined than the types of pneumococci which owe their specificity to the possession of reacting soluble substances peculiar to each type. Probably the antigenic structure of the streptococcus is similar to that of the meningococcus in which dominant and subsidiary antigens mark respectively the type and group characters of a strain. In my first report which deals with puerperal as well as scarlatinal streptococci, I have already discussed a possible analogy

from the epidemiological standpoint between the several disease groups of haemolytic streptococci and the whole meningococcal group, comprising strains from meningitis and from the normal nasopharynx.

It is possible that those strains of haemolytic streptococci found most commonly in scarlatinal throats, viz. Types I, II, III and IV, represent the most infective strains or those specially suited by their antigenic constitution for setting up scarlet fever. They are the equivalents of the epidemic types of meningococci. The heterogeneous scarlatinal strains, found also in puerperal septicaemia and in pyogenic infections, may rarely cause scarlet fever and perhaps never cause large outbreaks. They would stand in relation to scarlet fever in the same position as the nasopharyngeal meningococci amongst non-contacts do to cerebrospinal fever. That is to say, their infective qualities are feeble, and they give rise, the former to scarlatina and the latter to cerebrospinal fever, only in specially favourable circumstances.

The methods which I have employed in this investigation—direct agglutination combined with agglutinin absorption—do, I consider, reveal the dominant antigen which determines the type; they do not pretend to analyse the antigenic structure more closely. As a rule a simple direct agglutination test suffices to identify a strain belonging to one or other of the four types which have occurred most frequently in this series. Provided the strain has been recently isolated from the patient and forms a reasonably fine stable suspension, a clear cut result can be obtained by mixing on a slide a small loopful of undiluted serum of each of the four types with drops of the suspension.

Classification of those scarlatinal strains which do not fall under any of the four types is less straightforward, and agglutinin absorption is necessary to decide whether a strain agglutinated is identical with or at least possesses the same dominant antigen as that which produced the agglutinating serum.

The sera from these unclassified strains may have a wide range of action or may agglutinate only the homologous strain. The former wide range sera may agglutinate one strain of a type and yet not another of the same type.

Though at first sight the above result might open the question of the validity of the classification, it may be explained on lines which are not inconsistent with it. For example, a strain, No. 16, which possesses a highly individual antigen and produces a serum of the same character, agglutinates a strain of Type I. In the Type I strain there is a subsidiary antigen which reacts with the antibody to the dominant antigen in No. 16, though the amount of this subsidiary antigen is too small to produce sufficient antibody in the Type I serum to cause appreciable flocculation of No. 16.

There is thus a further analogy between the streptococcus and meningococcus groups, since certain nasopharyngeal meningococci which were agglutinated only slightly by the sera of meningococcal Types I and II, nevertheless produced in rabbits sera of a high titre which agglutinated both Types I and II as powerfully as they did the homologous.

Support for the validity of the classification as well as for the causal relationship of the haemolytic streptococcus to scarlet fever is provided by the finding of the same type in two or more members of one family in which scarlatina has occurred. Smith¹ has made similar observations. This fact is of importance in the investigation of outbreaks.

Serological classification is an essential step in the study of every bacterial group and it is of particular interest in the case of the haemolytic streptococcus which is associated with various forms of disease. Since 70 per cent. of scarlatinal streptococci have been found to fall into four types the identification of a strain as being of scarlatinal origin is rendered more feasible. The ability of a strain to form a Dick toxin might be taken as a criterion of scarlatinal origin, but, even if there was a simple method of identifying the specific properties of the toxin and if only scarlatinal strains produced it, a positive result would show only that the strain might conceivably cause scarlet fever. The advantage of serological identification is that it gives some indication of the degree of epidemic pathogenicity (shown by the frequency of occurrence) and apparently of the character of the disease the strain might set up. This latter advantage is clearly shown from the results of Dr W. Gunn's analysis of the clinical data in the cases from the Park Hospital; he found that the Type II strains in this series had generally produced a more severe form of scarlatina than the Types III and IV, or the unclassified strains.

There are many problems to the solution of which a reliable classification of scarlatinal streptococci will be of assistance. It opens the way to more intimate knowledge of the interrelationship between the various forms of streptococcal infections. It may also have to be taken into account in the manufacture of Dick toxin and antitoxic serum, both for treatment and for the Schultz-Charlton reaction.

Reference has been made above to the importance of serological types of streptococci in the investigation of outbreaks of scarlatina. I have found recently that an enquiry into the probability of books conveying scarlatinal infection was much simplified by a knowledge of the commoner types of scarlatinal streptococci. Haemolytic streptococci were grown from books which had been used by children prior to the development of scarlet fever, and while several cultures gave no indication of their probable origin two of the fixed types were found. In the case of one of the latter it was possible to obtain from the child the same type of scarlatinal streptococcus, Type II, as that which was obtained from the books which the child had used.

SUMMARY.

A series of 222 strains has been analysed serologically and 156 have been found to react with one or other of four sera prepared with haemolytic streptococci of scarlatinal origin.

Among the 156 strains the four chief types were distributed in the

¹ Smith, J. (1926). *J. Hygiene*, 25, 165-175.

following proportions: 10 of Type I, 57 of Type II, 45 of Type III and 44 of Type IV.

The remaining 66 strains of the series have not yet been classified; though they certainly include individualistic strains, there may be other types to be defined by further analysis.

The four chief types are well defined and can generally be identified by direct agglutination. Cross-agglutination may cause confusion when suspensions are unstable, in which case resort must be made to agglutinin absorption. In addition, certain Type I strains may also agglutinate with Type III serum.

Analysis of clinical data in a series of 100 cases shows clearly that a fairly severe form of scarlatina with greater tendency to complications is often caused by infection with Type II scarlatinal streptococcus; Type II has so far been found only in association with scarlet fever.

There were nine instances where two or more members of the same family developed scarlatina; in six instances the same type was found in each patient and in three each patient yielded an unclassified strain. In one instance the strains from two members of the same family were different.

Haemolytic streptococcus colonies on fresh blood agar present three chief varieties; there are minor differences between these dependent on the amount of moisture in the medium.

There is a certain correlation between variety of colony and serological type. Type III colonies retain a soft consistency after two days' incubation, while the other types generally become tough and can be lifted from the medium as a whole.

Rough and smooth colonies with differential characters analogous to those occurring in pneumococcus cultures have not been identified. An interesting change in appearance is the production of a clear dome-shaped watery or slightly mucinous colony (3rd variety) in a culture which ordinarily forms either more or less opaque or soft coherent colonies.

There is evidence that the proportions of the four main types are likely to vary in different localities and in different outbreaks of scarlet fever in the same locality.

The importance of a reliable classification of haemolytic streptococci is discussed.

(MS. received for publication 27. iv. 1927.—Ed.)