STUDIES ON THE SEROLOGICAL TYPING OF STREPTOCOCCUS HEMOLYTICUS*

By RUTH H. PAULI and ALVIN F. COBURN, M.D.

With the Assistance of Florence Stone, Ph.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

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Two factors involved in the development of rheumatic activity have seemed especially significant. Recent emphasis has been placed on the character of the immune response of the rheumatic subject to respiratory infection with hemolytic streptococcus. Swift and Hodge (1) have found a delay in the appearance of anti-M precipitins in rheumatic patients as compared with non-rheumatic subjects. Our findings (2) have indicated that initiation of the rheumatic attack is associated with an immune response which is atypical in several respects.

The other factor in which we have been particularly interested is the nature of the infectious agent. The biological character of the infecting strain has seemed to determine in part whether or not the quiescent rheumatic subject develops a recrudescence following pharyngitis with hemolytic streptococcus (3). Preliminary observations have suggested that serological classification of these organisms may make it possible to identify the strains which are effective in initiating rheumatic activity from year to year in different sections of the world. The present studies deal first with the methods devised for simplifying the serological classification of hemolytic streptococcus and second with the results of typing of these organisms associated with pharyngitis in rheumatic subjects.

A systematic classification of hemolytic streptococcus of human origin was begun by Dochez, Avery and Lancefield (4). These authors

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found that 125 strains associated with respiratory infections could be subdivided by means of the agglutination reaction into at least four distinct biological types. Lancefield (5) demonstrated the presence of a type-specific substance of protein character in hemolytic streptococcus and developed a method for classification based on the precipitin test. Coburn and Pauli (6), applying the precipitin method of Lancefield to strains of hemolytic streptococcus recovered from the throats of rheumatic subjects during pharyngitis, found a number of different serological classes of hemolytic streptococcus. Complete serological classification of these organisms was impracticable at that time because of the cross-reactions due to the high concentration of carbohydrate antibody (anti-C) in some of the rabbit antisera (6). The removal of anti-C by absorption was attempted, but had to be abandoned as repeated absorptions with a crude fraction of streptococcus carbohydrate diluted the content of precipitin to such a degree that the sera became useless.

A method recently developed by Griffith (7) in which slide agglutinations are performed with absorbed antisera has made it possible to distinguish more than twenty serological types of hemolytic streptococcus. This method permits type identification in a large majority of organisms and should be invaluable in epidemiological studies were it not handicapped by the technical difficulties in the absorption procedure. This step in Griffith's technique consists in absorbing each antiserum with a suspension composed of organisms from all of the heterologous types. The possibility of under- or over-absorption presents a constant danger to most workers. The present authors have therefore attempted to eliminate this difficulty in order that Griffith's method may have the widespread application which it deserves.

Comparative tests of Griffith's slide agglutination and Lancefield's precipitin methods have shown general agreement (8). In Lancefield's opinion both of these phenomena represent reactions between anti-M and the type-specific M substance. Since the presence of anti-C in rabbit sera is known to interfere (6) with satisfactory typing by the precipitin method, it seemed likely that it might also be responsible for cross-reactions in Griffith's method. The present paper deals with observations on typing with rabbit antisera from which anti-C had been removed.

Technique

1. Preparation of Broth Used in Making Antigens for Immunization.—2 pounds of chopped fresh beef hearts are placed in 2 liters of distilled water, extracted for 1 hour and refrigerated overnight. The mixture is boiled for 5 minutes, filtered through cheese cloth and the broth heated again to boiling. The pH is adjusted to 8.0. After bringing to boil, the volume of 2 liters is restored with boiling water. The pH is again checked. After 2 minutes of boiling, 20 gm. of neopeptone and 8 gm. Na_2HPO_4 are added. The broth is autoclaved for 25 minutes at 15 pounds pressure.

2. Preparation of Broth Used in Making Bacterial Suspensions.—400 gm. of fresh beef heart are minced. To this are added 1 liter of tap water, 0.5 gm. Na_2HPO_4. This is made alkaline to litmus with ½ normal sodium hydroxide. The mixture is heated to 70–80°C. for 5 minutes. After cooling to 42°C., bactotrypsin (Difco) is added, 10 cc. per liter. This is incubated at 45°C. from 1½ to 2 hours depending on the activity of the trypsin. Digestion is stopped when the biuret test becomes positive. The medium is acidified with concentrated HCl until it is distinctly acid to litmus. The medium is then boiled for a total of 20 minutes. At the end of 10 minutes of boiling, the acidity to litmus is tested and more HCl added if necessary. The medium is then filtered through muslin and the pH of the broth adjusted to 7.8. Sodium chloride is added to a concentration of 0.25 per cent and then calcium chloride to 0.13 per cent. The broth is then boiled for 5 minutes and filtered while hot through a fine filter paper (Eimer and Amend No. 23154). The filtrate is steamed in the Arnold sterilizer for 1 hour and for ½ hour on 2 successive days.

3. Preparation of Antigen for Immunization.—1½ liters of neopeptone buffered broth (described under 1 above) are inoculated with 5 cc. of a 5 hour seed culture (purity of culture being checked by Gram stain and plating). After 15 hours incubation at 37.5°C., the culture is cooled under running tap water and immediately centrifuged. The broth is decanted and the organisms are washed three times in 250 cc. normal saline. After the third washing, the bacteria are suspended in 250 cc. normal saline and immediately heated for 1 hour at 56–58°C. After centrifuging again, the killed organisms are suspended in 100 cc. of saline containing merthiolate (1 in 10,000). The vaccine is then standardized in concentration of 1, 2 and 5 billion organisms per cc.

4. Immunization Procedure.—Young male brown rabbits weighing 4 to 6 pounds are selected for immunization. A preliminary bleeding is made for control purposes. Vaccine is administered according to the following dosage.

1st week

1st	day	subcutaneously	0.5 cc. of 5 billion organisms per cc.
2nd	"	"	0.5 " " 5 " " " "
3rd	"	"	0.5 " " 5 " " " "
4th	"	intraperitoneally	1.0 " " 1 " " " "
5th	"	"	1.0 " " 1 " " " "
6th	"	"	1.0 " " 1 " " " "

2nd week					
1st day	intravenously	0.5 " " 1	"	"	" "
2nd "	"	0.5 " " 1	"	"	" "
3rd "	"	1.0 " " 1	"	"	" "
4th "	"	1.0 " " 1	"	"	" "
3rd week					
1st day	intravenously	2.0 " " 1	"	"	" "
2nd "	"	2.0 " " 2	"	"	" "
3rd "	"	1.0 " " 1	"	"	+ 0.05 cc. of living culture*
4th "	"	1.0 " " 1	"	"	+ 0.05 " " " "
4th week					
1st "	"	1.0 " " 1	"	"	+ 0.1 " " " "
2nd "	"	1.0 " " 1	"	"	+ 0.1 " " " "
3rd "	"	1.0 " " 1	"	"	+ 0.1 " " " "
4th "	"	1.0 " " 1	"	"	+ 0.1 " " " "

5th week

1st day—trial bleeding and testing.

2nd day—bleeding if titer is satisfactory. After large amounts of blood are taken, a 5 per cent glucose infusion is given and rabbits are allowed to rest for 4 or 5 weeks, after which they are given one series intravenously of vaccine (1 or 2 billion organisms per cc.). Upon testing, it will be found that in most instances the titer has risen, and the rabbits are then suitable for further bleeding.

If the titer at first testing is found too low, further immunization is done for 2 weeks. Should the rabbits not respond, they are discarded and new rabbits immunized.

* All living cultures are washed three times and made up to original volume with normal saline.

5. *Technique of Slide Agglutination.*—The technique for testing the antisera and for identifying the type of unknown organisms is the same. We have followed Griffith's method in detail. 10 cc. of a 15 hour trypsin broth (described above under 2) culture are thoroughly mixed in a 10 cc. pipette. The culture is then centrifuged and most of the broth decanted. The organisms are suspended evenly in the remaining broth (approximately 0.1 to 0.2 cc.) by repeated aspiration into a capillary pipette. It is important that the suspension be homogeneous. A small drop of this bacterial suspension is placed on a slide with the capillary pipette and a tiny loopful (nichrome wire gauge 34, diameter of loop 0.5 mm.) of rabbit antiserum in the appropriate dilution is mixed with the suspension on the slide. The slide is rotated and readings are made immediately with a hand lens (magnification 6 ×). The results are read as follows:

++++ drop containing large clumps which tend to settle around the periphery of drop, making a heavy outline.
+++ similar to the above except that the clumps are not so heavy.
++ many fine clumps of agglutinated organisms.
+ few small clumps of agglutinated organisms scattered throughout the suspension.
0 homogeneous suspension—no clumps.

Examples of each are illustrated in Fig. 1.

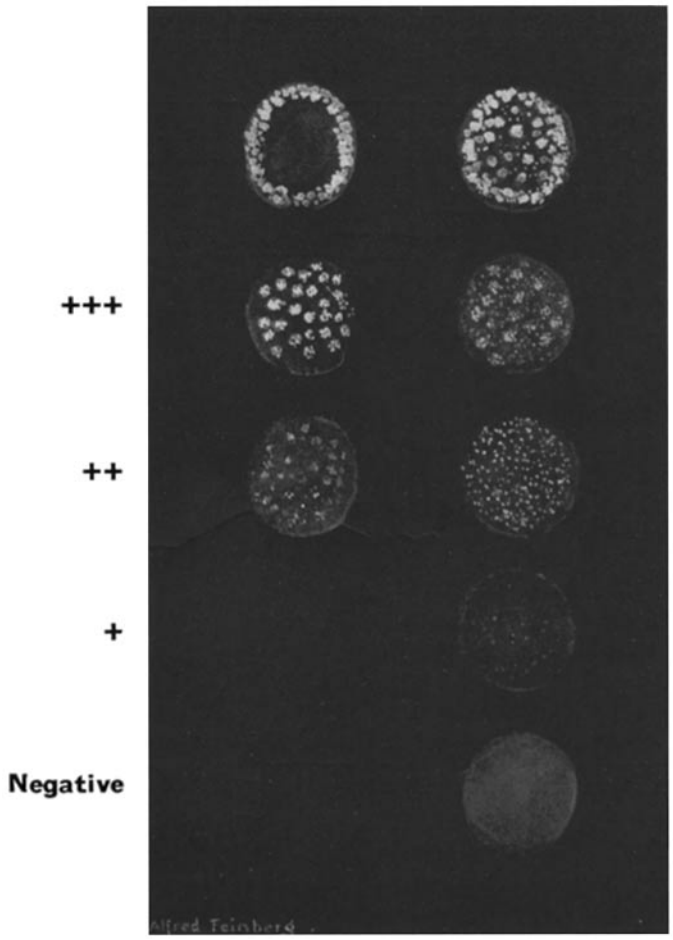
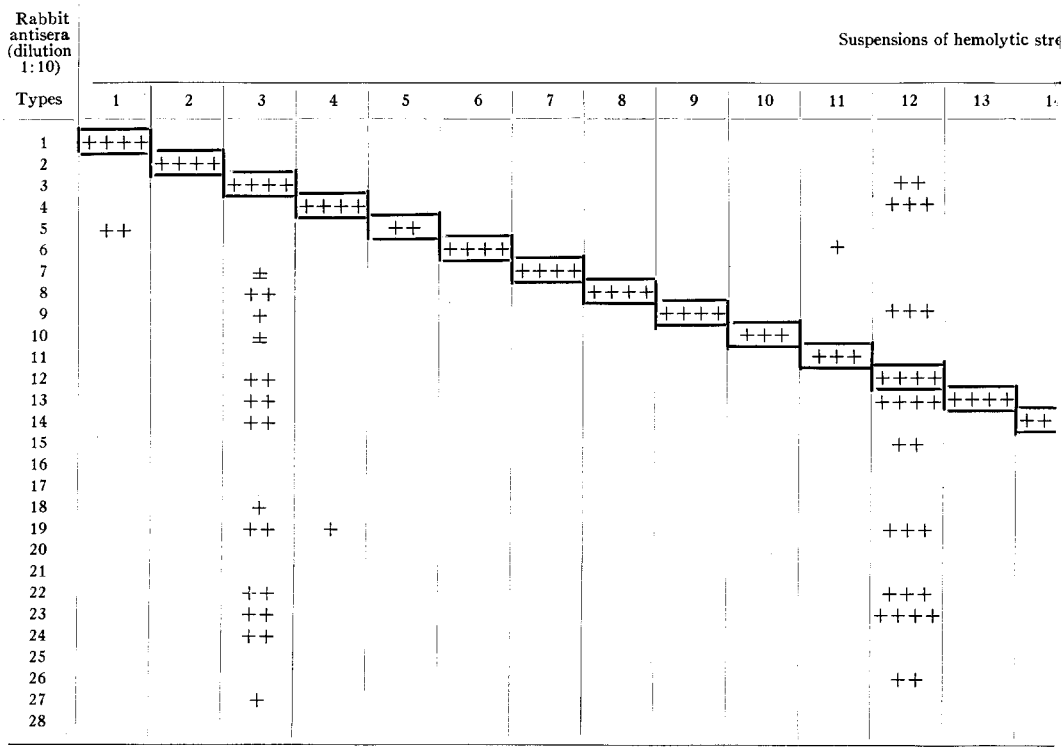


FIG. 1

TABI

Slide Agglutination Reactions between Crude Rabbit Antisera a

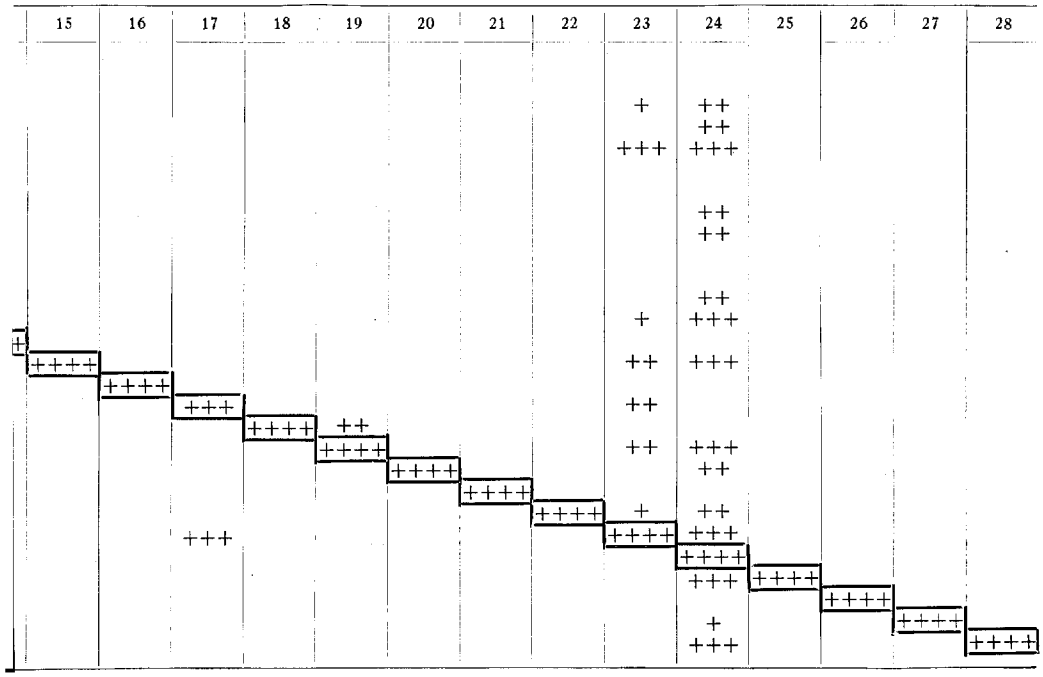


The blank spaces in the tables indicate negative results.

E I

l Suspensions of the Homologous and Heterologus Organisms

tococcus—Griffith's 28 type strains



The above technique is essentially that used by Griffith. However, two special precautions have been introduced in order to minimize cross-reactions without resorting to absorption. First, the cultures used for immunization were washed in saline at least three times to free them from broth and to remove any carbohydrate which might adhere to the surface of the bacterial cells. Second, the rabbits were bled as early as possible in the course of immunization (4th week) in order to obtain sera with minimal anti-C titers. The agglutination reactions between the 28 crude antisera and Griffith's 28 type strains are presented in Table I.

There are three points of interest in Table I. First, five of the antisera were type-specific: that is, they agglutinated only the suspension of homologous organisms (*e.g.* types 1, 2, 7, 11 and 21).

TABLE II

Relation between Anti-C Content and Intensity of Cross-Reactions in Rabbit Antisera

Unabsorbed rabbit antisera	No. of cross-reactions with cell suspensions of heterologous types	Precipitin reactions with streptococcus carbohydrate dilution 1:10,000	
		2 hrs.	24 hrs. (centrifuged)
Type 1	0	0	(0)
Type 2	0	0	(0)
Type 15	Four (++)	+	(++±)
Type 23	Four (+++)	+++	(++++)

Second, a number of antisera showed cross-reactions of varying degree with suspensions of heterologous types; slight (type 3), moderate (type 22), marked (type 13). Finally, four of the bacterial suspensions (types 3, 12, 23, 24) were agglutinated by a number of antisera.

The Relation of Anti-C to Cross-Reactions

In order to test the possibility that cross-reactions might be due to the presence of anti-C in the sera, the concentration of this antibody was determined in antisera of the various types. The test was performed by adding 0.2 cc. of crude antiserum to 0.2 cc. of purified carbohydrate[1] diluted 1:10,000. Four observations of precipitin

[1] The streptococcus carbohydrate fraction used throughout this study was kindly given to us by Dr. Michael Heidelberger and Dr. Forrest E. Kendall. The method of preparation is to be reported by them.

TABLE III

The Effect of Repeated Absorptions with Carbohydrate on the Specificity of Slide Agglutination

	Type No.	Precipitin reaction with carbohydrate				Slide agglutination reactions with suspension of types			Reactions of unabsorbed controls diluted with saline
						3	12	24	
Reactions of unabsorbed undiluted sera	3	0	±	+±	(++++)	++++	++++	++++	
	12	+++	+++	++++	(++++)	+++	++++	+++	
	24	+	++	++++	(++++)	+++	++++	++++	
Reactions after first absorption of crude sera	3	0	0	±	(±)	++++	++	+++	Same as original sera
	12	0	±	++	(++)	++	++++	+	
	24	0	±	++	(++)	++	+++	++++	
Reactions after second absorption	3	0	0	0	(±)	++++	+	0	Same as original sera
	12	0	0	±	(+±)	++	++++	+	
	24	0	0	+	(++)	++	+++	++++	
Reactions after third absorption	3	0	0	0	(0)	++++	±	±	Same as original sera
	12	0	0	0	(++)	+	++++	+	
	24	0	0	±	(++)	++	+++	++++	
Reactions after fourth absorption	3	0	0	0	(±)	++++	0	±	Sera showed slight diminution in strength of heterologous reactions
	12	0	0	0	(+)	+	++++	+	
	24	0	0	±	(++)	0	++	++++	

formation were generally made: immediately, after 2 hours in 37° water bath, after refrigeration overnight and after centrifuging. A sample protocol is presented in Table II.

As illustrated in Table II, type-specific antisera were free of anti-C and the sera which gave cross-reaction contained anti-C. This was observed throughout the 28 types. In general there was a close correspondence between the concentration of anti-C and the intensity of cross-reactions.

The sera containing anti-C were absorbed with carbohydrate. The number of absorptions necessary was found to vary directly with the concentration of anti-C. However, repeated absorptions caused no apparent diminution in the strength of the type-specific agglutination reaction. This is shown in Table III. From this it is seen that repeated absorptions progressively diminished the cross-reactions without at all weakening the homologous reactions.

The next step consisted in a simplification of the absorption technique. It was found that by diluting the serum 1:5 and absorbing

TABLE IV

The Elimination of Cross-Reactions

Treatment of type 23 serum	Reactions with carbohydrate				Reaction with suspension types			
					3	12	23	24
Unabsorbed, diluted 1:10	+	+++	++++±	(++++)	+	++	++++	++++
Diluted 1:5 and absorbed with equal volume of carbohydrate 1:5,000. Final dilution 1:10	0	0	0	(0)	0	0	++++	0

with an equal volume of carbohydrate 1:5,000 a single absorption was usually sufficient to eliminate cross-reactions. This is illustrated by Table IV.

It is to be observed from Tables III and IV that the absorption of anti-C was paralleled by the disappearance of cross-reactions, irrespective of the number of absorptions and the dilutions employed.

Removal of anti-C in no case weakened the type-specific reaction. The following procedure has been adopted as standard in this laboratory. Antisera which contain no anti-C and give no cross-reactions are simply diluted 1:10 without absorption. The antisera which contain anti-C and give cross-reactions from + to +++ are diluted 1:5 and then absorbed with an equal volume of carbohydrate, 1:5,000. After the precipitate is removed, the supernatant is a type-specific serum and has a final dilution of 1:10. The antisera which contain a great deal of anti-C and give strong cross-reactions are diluted 1:2.5 and absorbed with an equal volume of carbohydrate 1:5,000. The supernatant is then reabsorbed with an equal volume of carbohydrate 1:10,000. This doubly absorbed serum is either type-specific or may give insignificant cross-reactions which can be removed if desired by further absorption. The results of the slide agglutination tests with the antisera treated in this way are presented in Table V. The final dilution of all sera is 1:10.

Table V shows that after the sera had been absorbed with carbohydrate, only four cross-reactions persisted. Two of these were readily removed with one more absorption. However, it was found that type 17 serum freed of all anti-C gave a cross-reaction with type 23 organisms, and that type 23 serum freed of all anti-C gave a cross-reaction with type 17 organisms. No other sera gave cross-reactions after anti-C had been completely removed. It appeared from this slide agglutination that these two types were antigenically indistinguishable. Two further attempts to differentiate these sera were made. First, each serum was absorbed with the homologous and the heterologous organisms and then tested for agglutinins to types 17 and 23. Again the two sera were indistinguishable. Finally sera of the two types were tested for precipitins to type-specific M substance (5). No differences could be detected between types 17 and 23.

With the procedure as outlined it is possible to obtain strong agglutinating antisera in 1 to 10 dilution that are, for practical purposes, type-specific. Such sera may be pooled (type 1 to 6, 7 to 12, etc.). With this preliminary step the organism is found to be one of five groups. Type identification may then be made quickly and simply.

TAE

Slide Agglutination Reactions between Rabbit Antisera Absorbed with

Absorbed sera in 1:10 dilution	Suspension of hemolytic str												
Types	1	2	3	4	5	6	7	8	9	10	11	12	13
1	++++												
2		++++											
3			++++										
4				++++									
5					+++								
6						++++							
7							++++						
8								++++					
9									++++				
10										+++			
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LE V

arbohydrate and Suspensions of Homologous and Heterologous Organisms

ptococcus—Griffith's 28 type strains

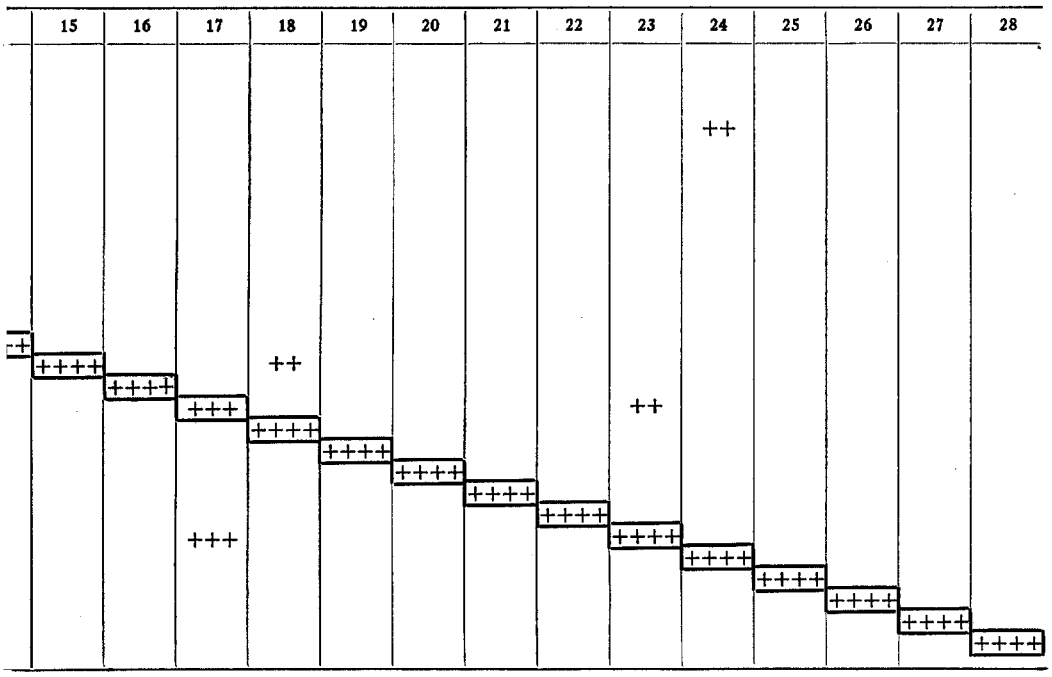

TABLE VI

Slide Agglutination Reaction of Bacterial Suspensions Types 12 and 24

Type of antiserum.....	3	4	5	8	9	12	13	15	19	20	22	23	24	25	26	27	28	All others
Type 12 Organisms grown in standard way	++	+++	0	0	+++	++++	++++	++	+++	0	+++	++++	0	0	++	0	0	0
Type 12 Organisms grown in presence of anti-C sheep serum	0	0	0	0	0	++++	0	0	0	0	0	0	0	0	0	0	0	0
Type 24 Organisms grown in standard way	++	++	+++	++	++	++	+++	+++	+++	++	++	+++	++++	+++	0	+	+++	0
Type 24 Organisms grown in presence of anti-C sheep serum	0	0	0	0	0	0	0	0	0	0	0	0	++++	0	0	0	0	0

As was seen in Table I, some of the bacterial suspensions showed a tendency to be agglutinated by many antisera of heterologous types. This was most marked in the case of type 12 and 24 organisms. One possible explanation for this phenomenon is that these bacterial cells are rich in carbohydrate. With a view to inhibiting carbohydrate formation these organisms were grown in broth containing 10 per cent sheep antistreptococcus serum. This sheep serum had a high titer of antibody to the carbohydrate; that is, it gave a ++++ reaction uncentrifuged. Daily transfers of minimal inoculations of an 18 hour culture were made for 2 weeks. At the end of that time the suspensions of organisms showed a tendency to become granular but were nevertheless readily typed. The resulting improvement in specificity of one of these types is shown in Table VI.

When grown in the usual manner these organisms consistently reacted with the sera of the heterologous types containing anti-C. However, the bacterial cells prepared by prolonged culture in anti-C sheep serum broth reacted only with the homologous rabbit antiserum. The authors are now attempting to obtain antisera which give only type-specific reactions by immunizing with organisms in which the formation of carbohydrate has been repressed.

Results of Typing 1935–1936 Cultures

147 strains of hemolytic streptococcus were obtained from throat infections between September, 1935, and August, 1936. Of these 107 were identified and the types were distributed as follows:

Type	1	2	3	4	5	6	7	8	9	10	11	12	13	14
No. of cultures	3	2	9	26	4	5	4	0	2	0	0	0	17	0
Type	15	16	17*	18	19	20	21	22	23	24	25	26	27	28
No. of cultures	0	6	4	4	1	0	2	15	0	0	1	0	1	1

* These four suspensions gave strong agglutination reactions with type 17 antiserum, weak reactions with Griffith's provisional type 23 antiserum and are classified as type 17.

Most of these strains were sent to Dr. F. F. Griffith for independent classification. Using his own suspensions and antisera, he checked every typing but one.

40 strains could not be typed by the standard procedure, for two reasons: (*a*) Certain strains were too granular for the slide agglutination technique. This may have been due to some unexplained characteristic of particular batches of trypsin broth, or to some characteristic of the organism. A tendency to be granular was especially noticeable in organisms which formed extremely matt colonies. The difficulty could be overcome in some instances by rapid passage through trypsin broth (every 3 hours), followed by plating on blood agar. A smooth colony was then selected for replanting in broth. Dr. Griffith succeeded in identifying twelve of these refractory strains. (*b*) Four other strains gave excellent suspensions but were not agglutinated by any of the available antisera. It is possible that these organisms either lack specific M substance, or belong to types as yet unidentified.

The Relation of the Types to Rheumatic Recrudescences

The above strains were obtained from throat infections in three groups of patients, all resident in or near New York City. One group of ten had scarlet fever; the predominant organism was type 3. Another group consisted of nurses with tonsillitis; these yielded a variety of types. The largest group consisted of rheumatic subjects from whom three predominant types were recovered: 13, 4 and 22.

It is our impression that the effects of strains 22 and 13 on rheumatic subjects were clinically different. Type 22 seemed relatively ineffective in initiating rheumatic activity. In contrast, most of the recrudescences following pharyngitis with type 13 were especially severe (2). It will be of interest to learn whether these differences between types are constant from year to year and whether new types will appear in predominance. Similar studies in other environments should furnish valuable information.

DISCUSSION

Lancefield's (9) serological classification of the groups of hemolytic streptococcus makes it possible to identify strains pathogenic to man (group A). Further subdivision in this group A can be accomplished with Griffith's technique. It seems to us that some of the types originally defined by Griffith must be eliminated. Four of these are 7, 16, 20 and 21 which do not belong to group A and should be

classified in their appropriate groups. Type 23 in our experiments is indistinguishable from Type 17 and we believe that both have the same type-specific antigen. As new types appear in human infections their group should be determined and those which belong to group A should be given type numbers in Griffith's series, beginning with those numbers which have become available by the above or any future elimination of provisional types.

The slide agglutination reaction, like the precipitin reaction, depends on the presence of either or both of two substances. Group-specific reactions occur in the presence of C substance and its antibody and type-specific reactions occur in the presence of M substance and its antibody. These two antigens must, therefore, be present on the surface of the bacterial cells. There is another antibody which we know is present in high titer in our rabbit antisera but which does not take part in the slide agglutination reaction. This is anti-P. The simplest explanation for the absence of reaction between bacterial suspensions and anti-P is that P is not present on the surface of the bacterial cell.

SUMMARY

The cross-reactions which interfere with satisfactory serological identification of hemolytic streptococcus are due to anticarbohydrate in the sera used for typing.

This antibody can be removed easily by absorption with purified streptococcus carbohydrate, and type identification is then readily established.

The serological classification of hemolytic streptococcus from throat infections contracted in New York during 1935 and 1936 showed the predominance of types 4, 13 and 22. Type 13 appeared to be the most serious in initiating rheumatic activity during this period of observation.

The authors are deeply indebted to Dr. F. F. Griffith of the British Ministry of Health for invaluable assistance and to Dr. Forrest E. Kendall for advice throughout this study.

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