DIFFERENTIATION OF GROUP A STREPTOCOCCI WITH A COMMON R ANTIGEN INTO THREE SEROLOGICAL TYPES, WITH SPECIAL REFERENCE TO THE BACTERICIDAL TEST

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The classification of group A streptococci into types depends upon specific proteins, the M antigens, characteristic for each type. These M antigens are associated with virulence, and streptococci which contain them give rise to antibodies which promote phagocytosis of streptococci of homologous type and lead to type-specific protection of infected animals. Anti-M antibodies are demonstrable by M-precipitin reactions, bactericidal tests, and active and passive protection tests in mice (1–8).

In studying the M proteins, it was found that the protein antigen which served to identify strains designated by Griffith as type 28 differed from the type-specific antigens of all other types in that it was not inactivated by trypsin and several other proteolytic enzymes (9). Pepsin was the only enzyme found which destroyed this antigen. Further studies showed that strains containing this antigen did not represent a single type, and that the antibody which had been used as the basis for their classification into one type did not protect mice against infection with these strains. Since this trypsin-resistant protein, now designated as R antigen, lacked one of the most characteristic properties of type-specific M antigens, namely, the ability to stimulate protective antibodies, it was obviously not the type-specific substance. Additional investigation of these strains was therefore undertaken (10).

Initial attempts to classify two of these R-containing strains yielded inconclusive results: mouse protection and precipitin tests for M antibody in antiserum prepared against each were not definitive but suggested that, although partial cross-protection occurred, one strain might belong to type 2 and the other to type 13.

In order to clarify these relationships it was thought possible that bactericidal tests might reveal specific M antibodies more readily than the tests used previously. The bactericidal method depends upon the phagocytosis and destruction of streptococci by human leukocytes in the presence of M antibody. The results obtained have the same significance with regard to immunity as those

obtained with mouse protection tests. This test has the advantage that the streptococci need not be virulent for mice. It is essential only that the strains contain enough M antigen to permit growth in normal human blood under the conditions of the bactericidal test. In the present study R-containing strains were separated by means of bactericidal tests into 3 serological types: types 28, 2, and a third type closely related to type 13 but not identical with it. The latter is designated as type 48. These results were confirmed by precipitin reactions.

The data presented also serve to illustrate, in the same way as Maxted's recent work (11), the value of the bactericidal test as a means of determining the occurrence and specificity of M antigens in group A streptococci and of the corresponding anti-M antibodies in antistreptococcal sera. With the bactericidal test, type specificity can be established as conclusively as with the standard mouse protection test.

Methods

Streptococcal Strains:

Fifty-nine strains of group A streptococci containing R antigen were studied. These cultures had been isolated in England, Canada, Australia, and various parts of the United States.[1] For purposes of comparison, group C streptococci containing R antigen, and members of types 2 and 13 which did not contain R antigen were included in this study. In addition a few strains without R antigen were found which belonged to type 48, newly described below.

Antisera:

These were prepared in rabbits as already described. Absorption techniques were those used previously (2, 12, 13). Antisera containing R antibody were also absorbed with R-containing strains of heterologous serological types until free of R antibody as determined by precipitin reactions.

During the course of immunization with R-containing streptococci, the anti-M titer of the rabbit serum was followed by use of indirect bactericidal tests. This proved more convenient in preliminary testing than the precipitin reaction which requires removal of anti-R antibodies by absorption.

Immunological Reactions:

A. Precipitin Test.—The capillary technique was the chief method employed for the precipitin test (14). Special tests were performed in larger volumes either in ring tests or mixed in small test tubes (12). A constant amount of serum was mixed with varying dilutions of antigen. Streptococcal extracts were prepared by the hydrochloric acid method and, in the case of R antigen, by tryptic digestion of the bacteria or by heating bacterial suspensions at pH 7.8 (10).

B. Slide Agglutination.—Griffith's method, or a slight modification of it, has been used

[1] I am particularly indebted to Dr. Elaine L. Updyke, Communicable Disease Center, Chamblee, Georgia, for sending me 33 strains referred to the Diagnostic Unit of the Public Health Service from many parts of this country.

(1). Some antisera were obtained from the Streptococcus Reference Laboratory, Colindale, England;[2] others were prepared in this laboratory and absorbed especially for slide agglutinations tests (15).

C. The Bactericidal Test.—

1. General considerations: This test is essentially the same as that developed by Todd in 1927 for studying the bactericidal effect of the blood of patients on the homologous hemolytic streptococcus (16). More recently several investigators have used this test, slightly modified, for studies of the type-specific antibody response of patients following infection with group A streptococci (4, 5, 7, 17–19). Lyons and Ward also suggested in early studies the use of a similar test, based on the opsonic index, for type classification of group A streptococci, but they were unable to overcome the difficulties connected with the test (7).

(*a*) *Apparatus.*—Glass tubes, 90 × 8 mm. were used as containers. They were closed with tightly fitting soft rubber stoppers with a smooth lower surface. The bottom of each stopper was coated with a thin layer of silicone (1:100 dilution in benzene of Dow-Corning No. 550 silicone) which did not interfere with the test. The silicone makes it possible to force a tight, freshly boiled stopper into the tube, but if more than a minimal amount is used, the stoppers may work out of the tubes during incubation.

(*b*) *Antibody.*—For the direct bactericidal test, antibody and phagocytes are both supplied by the same blood. For the indirect test rabbit sera, or occasionally human sera or plasma, were used as the source of antibodies. Fresh sera were heated for ½ hour at 56°C.; old sera were unheated. The addition to the rabbit serum of merthiolate, 1:10,000 final concentration, did not affect phagocytosis and was used in some experiments. The final concentration of merthiolate in the test was never more than 1:40,000 and was usually less.

(*c*) *Phagocytes.*—Attempts to use normal rabbit blood as the source of phagocytes for group A streptococci were unsuccessful except for a single rabbit. Most other investigators have found rabbit leukocytes greatly inferior to human leukocytes in this test even when rabbit immune serum was used. Stollerman and coworkers have recently reported encouraging results with rabbit leukocytes but not yet sufficiently consistent to substitute rabbit for human leukocytes (20). Adult human blood was, therefore, used for both direct and indirect tests, with heparin as the anticoagulant (heparin sodium, Abbott, containing 1000 units/cc. was used in the proportion of 0.1 cc. for 20 cc. of human blood or 0.2 cc. for rabbit blood). For the indirect test, adult blood is satisfactory if it does not contain antibodies for the types of streptococci being studied and is considered "normal" with respect to these types. The heparinized blood and the culture dilutions (see below) were kept cold and were used within 2 hours of preparation.

(*d*) *Preparation of cultures.*—The stability of streptococci with regard to M antigen varies considerably. Stable strains may be kept as stock cultures in rabbit blood broth in the refrigerator for 1 to 2 weeks. Less stable strains should be freshly prepared each day from lyophilized or frozen stocks. The supernatant fluid of an overnight culture in rabbit blood broth may be used, but it is preferable to prepare a 1½ to 2 hour rapidly growing subculture, heavily inoculated from the overnight culture. Tenfold culture dilutions proved too widely spaced and a useful series is furnished by starting with a culture dilution of 10^{-5}, further diluted serially 1:4, 1:16, 1:64, 1:128 in Todd-Hewitt broth. As noted by previous workers, the best results are obtained with small inocula.

2. The direct bactericidal test: 0.1 cc. of the serial culture dilutions and 0.3 cc. of heparinized patient's or normal blood are placed in a series of tubes, using 0.2 cc. Kahn pipettes for small volumes. The tubes are then stoppered and incubated at 37°C. with the least possible delay. During incubation, the tubes are rotated end over end in a mixing machine at

[2] I am indebted to Dr. R. E. O. Williams and Mr. W. R. Maxted for generously supplying me with these sera.

6 R.P.M., with 2 complete mixings at each revolution.[3] If the tubes are incubated without constant mixing, only a trace of phagocytosis occurs and no bactericidal effect is observed.

After 3 hours rotation, pour plates are made with 0.1 cc. of each mixture and agar containing 3 per cent rabbit blood. After overnight incubation the number of colonies is counted or estimated. In the present experiments, in order to increase the sensitivity of the test, this method of determining the results was preferred to streaking on the surface of blood agar plates, as described by Rothbard, although with strongly positive results the latter method is satisfactory (5).

3. The indirect bactericidal test: 0.05 cc. of clear serum or plasma is pipetted into the requisite number of tubes. Culture dilutions (0.1 cc.) and heparinzed normal human blood (0.3 cc.) are then added, and the same procedures followed as for the direct test. A preliminary period of contact between culture and antiserum before the addition of blood cells did not affect the results.

The number of bacteria inoculated into each tube at the beginning of the test is determined in pour plates containing 0.1 cc. of each of the serial culture dilutions mixed with rabbit blood agar. Parallel tests showed that the results obtained in this way are comparable to those obtained by plating 0.1 cc. directly from the final mixtures prior to rotation of the tubes.

4. Strains suitable for the bactericidal test: Strains of group A streptococci suitable for bactericidal tests need not be virulent for mice but they must produce readily detectable amounts of M antigen and they must also grow well in "normal" human blood. The susceptibility of such strains to phagocytosis by leukocytes is increased in the presence of type-specific antibodies. Many strains, if lyophilized or frozen at −70°C. shortly after isolation from patients have these properties. Others require special procedures to make them suitable. Although the lower limit of M antigen necessary is not known, it was found that any method that enhances the production of M antigen also increases the ability of group A streptococci to grow in normal human blood and, in addition, augments their susceptibility to phagocytosis in the presence of type-specific antibodies.

Glossy variants are, therefore, not suitable for use in the bactericidal test because they produce little or no M antigen and consequently grow poorly in human blood under the conditions of this test. Furthermore, phagocytosis of glossy variants is only slightly increased in the presence of type-specific antibodies.

An example of these differences is given in Table I. In the absence of antibody, the matt variant of strain S43 grows rapidly in normal human blood while the number of viable cells of the glossy variant decreases. On the other hand, type 6 antiserum causes a striking effect in promoting destruction of the matt variant by human blood but has only little effect on the glossy variant.

Methods for Increasing Production of M Antigen:

With cultures deficient in M antigen, random selection of matt or mucoid colonies from blood agar plates sometimes yields strains with increased M antigen which are, therefore, suitable for testing. In type 2, as well as some other types, good results have been obtained by fishing opaque colonies from cultures on blood agar plates containing 20 per cent normal rabbit serum (22). A low power dissecting microscope with transmitted light is employed. Griffith recommended plates containing 2 per cent type-specific immune serum for selecting opaque type-specific colonies (1). The use of crude hyaluronidase preparations for this pur-

[3] A rotating drum, such as the model designed for tissue culture roller tubes, is used in some laboratories and may be easier to manipulate than a rotator requiring accurately fitted rubber stoppers (21).

pose without added serum in the plates prevents mucoid growth due to hyaluronic acid capsules and at the same time supplies the necessary normal serum component (23).

If cultural procedures fail to increase M antigen, serial passage through mice often enhances both the virulence for mice and the production of M antigen. As soon as adequate amounts of M substance become demonstrable, the culture isolated from the last mouse in the series is lyophilized or frozen as quickly as possible.

TABLE I

Growth of Variants of Group A Streptococci with Different Amounts of M Antigen in Bactericidal Tests

(*a*) Ability to grow in normal human blood.

(*b*) Effect of adding type-specific antiserum.

Variants of type 6 strains		Results of bactericidal tests No. of colonies				
Matt variant with M antigen* (Strain S43/137)	No. of streptococci inoculated	*Inoculum*				
		2000	760	181	42	9
	No. of streptococci at end of test with normal human blood and	*Bactericidal tests*				
	(*a*) Normal rabbit serum‡	∞	∞	∞	2000	700
	(*b*) Type 6 antiserum	6	3	0	0	0
Glossy variant without M antigen* (Strain S43 G)	No. of streptococci inoculated	*Inoculum*				
		∞	2000	700	96	8
	No. of streptococci at end of test with normal human blood and	*Bactericidal tests*				
	(*a*) Normal rabbit serum‡	∞	1000	151	13	1
	(*b*) Type 6 antiserum	1000	192	11	3	2

* Variants derived from the same original strain.

‡ Tests without any added serum gave the same results as those with normal rabbit serum.

Technique of Indirect Bactericidal Test.—

Suitable serial dilutions of culture were made for the inoculum. 0.1 cc. of each dilution was plated in a blood agar pour plate to determine the number of streptococci inoculated.

Each tube of the bactericidal test contained:—

0.05 cc. serum or plasma.

0.1 cc. culture dilution (several dilutions were used for each test).

0.3 cc. heparinized normal human blood.

After 3 hours rotation at 37°C., 0.1 cc. sample from each tube was plated in blood agar pour plates and incubated overnight.

The number of colonies which grew out in the pour plates from inoculum and test were counted or estimated to determine the number of streptococci in each tube at the beginning and end of the test.

∞ indicates innumerable colonies with blood completely hemolyzed.

4000 indicates innumerable colonies with some areas of unhemolyzed blood. Records of 2000 to 500 colonies were estimated by comparison. Colonies on plates with <400 were usually counted.

See Methods for further details.

Although in most instances good results are obtained with serial mouse passage, occasionally, as noted by Todd (16 *c*) the ability of streptococci to grow in human blood is diminished rather than increased after mouse passage. This phenomenon was observed in the present studies with strain T28 after the 17th mouse passage. With this culture, both rabbit and mouse passage were tried. The virulence for mice was not increased even after 143 mouse and 10 rabbit passages, no type-specific M antigen could be found; and unless large inocula

TABLE II

Varying Effects of Animal Passage on Ability of Group A Streptococci to Grow in Normal Human Blood in Bactericidal Tests

Variants of strain T28		Results of bactericidal tests No. of colonies			
Before mouse passage	No. of streptococci inoculated	*Inoculum*			
		283	63	28	2
(Strain T28)	No. of streptococci at end of test with normal human blood and	*Bactericidal Tests*			
	(*a*) Normal rabbit serum*	∞	2000	250	200
	(*b*) T28/150A antiserum	6	5	9	0
After first mouse-passage series through 143 mice and 10 rabbits	No. of streptococci inoculated	*Inoculum*			
		∞	500	50	5
(Strain T28/143/Rb10)	No. of streptococci at end of test with normal human blood and	*Bactericidal Tests*			
	(*a*) Normal rabbit serum*	140	16	0	0
After second mouse-passage series through 150 mice	No. of streptococci inoculated	*Inoculum*			
		164	13	5	5
(Strain T28/150A)	No. of streptococci at end of test with normal human blood and	*Bactericidal Tests*			
	(*a*) Normal rabbit serum*	∞	2000	165	295
	(*b*) T28/150A antiserum	0	0	0	0

See Table I and Methods for techniques.

* The same results were obtained in tests without any rabbit serum.

were used, the culture no longer grew in normal human blood in bactericidal tests. A comparison of the bactericidal tests with these variants of strain T28 is recorded in Table II, and shows a marked decrease in the first mouse-and-rabbit passage strain in ability to grow in normal human blood. This part of the experiment was done in the presence of normal rabbit serum but the results were similar in experiments without rabbit serum.

A second series of mouse passages was started from the original culture, strain T28. In this second series, in contrast to the first, there was no diminution of the ability of this strain to grow in any normal human blood used in bactericidal tests, in spite of the fact that animal passage was continued for a total of 150 transfers. Furthermore, in this series, M antigen became readily demonstrable, although the culture remained avirulent for mice.

In attempts to increase the production of M antigen of a number of other cultures by serial mouse passage, a few failures similar to that encountered during the first series of animal passages with strain T28 were observed. The ability of these strains to grow in normal human blood was also diminished. A result of this kind, however, occurs only rarely.

EXPERIMENTAL

Identification of Certain R-Containing Strains as Members of Type 28.—The identification of five strains of group A streptococci as members of type 28 is shown in the results of bactericidal tests recorded in Table III. All of these strains contain R antigen. In the first part of the experiment (Table III (*a*)) the direct bactericidal method was employed. Blood taken 8 months after a throat infection of one of the human subjects, LA, with strain B574 was used in routine tests before satisfactory rabbit antisera were available. As the table shows, not only did the blood of this individual inhibit the growth of the homologous culture, but also that of strain Small originally isolated by Griffith and designated by him as type 28 on the basis of slide agglutination (24). In addition to these two strains, similar results were obtained with 3 other cultures, C294, C670, and B655. Bloods from two normal individuals, WE and MC, did not inhibit the growth of any of these strains.

These results were subsequently confirmed with the indirect test (Table III (*b*)) with rabbit immune sera.[4] With normal human blood as the source of phagocytes and rabbit antisera of types 28, 2, 13, and 48 to supply antibodies, it is evident that the growth of all 5 strains was inhibited by type 28 rabbit antiserum and not by antisera for types 2 and 13 or by normal rabbit antiserum. Antisera for type 48, described below, were also ineffective in tests with type 28 streptococci.

In order to compare the direct and indirect methods of demonstrating type-specific antibodies, the serum of the individual, LA, who had had an untreated pharyngitis due to strain B574, was tested by the indirect bactericidal test with leukocytes supplied by the blood of a normal adult of a compatible blood group. The inhibition of growth of strain B574 in this experiment was less marked than that obtained in the direct test with the blood of LA unless the amount of LA serum added was increased from the 0.05 cc. ordinarily used to the volume present in the whole blood. This was done by removing the plasma from the normal blood used in the test and substituting plasma separated from LA blood. With hyperimmune rabbit serum and normal human blood, however, antiserum in high dilution could be used. For example, rabbit antiserum diluted 1:125 was only slightly less inhibitory than undiluted serum.

In comparison with the high titers obtained with rabbit antiserum and normal human blood, the titer of type-specific opsonins which persist in human blood following naturally occurring infections is relatively low. More definite results with human antibodies are, therefore, usually obtained with the direct rather than the indirect bactericidal test.

Type-Specificity of the Bactericidal Test.—In order to test the specificity of the re-

[4] Maxted has reported recently that he could not obtain type-specific reactions with rabbit antisera and strain T28, a finding in accord with previous unsuccessful attempts made in this laboratory before the satisfactory antisera finally prepared from the second mouse-passage series of this strain (T28/150A) eliminated this difficulty (11).

TABLE III

R-Containing Streptococci Classified as Type 28

Bactericidal Reactions

*Direct and indirect tests		Type 28 strains tested																			
		T28/146A				‡B574				C294/154				C670				B655			
		Results of bactericidal tests No. of colonies																			
(*a*) Direct test	No. of streptococci inoculated	*Inoculum*																			
		132	10	1	0	400	40	4	1	173	17	2	1	275	28	3	1	524	53	5	1
Phagocytes and antibodies in same human blood	No. of streptococci at end of test with human blood:	*Direct bactericidal tests*																			
	(*a*) LA, 8 mos. after infection	211	16	0	0	50	21	0	0	56	1	0	0	36	8	1	0	148	25	8	1
	(*b*) We, normal	2000	500	79	47	∞	∞	750	99	∞	2000	400	25	2000	260	25	0	∞	1000	106	12
	(*c*) MC, "	2000	250	200	45	∞	∞	600	50	∞	∞	750	78	2000	1000	174	3	∞	1000	128	10
(*b*) Indirect test	No. of streptococci inoculated	*Inoculum*																			
		300	30	3		324	81	18	0	165	25	9	2	150	48	10	5	400	76	8	2
Phagocytes in normal human blood and serum from immunized or normal rabbits	No. of streptococci at end of test with normal human blood and	*Indirect bactericidal tests*																			
	(*a*) Type 28 rabbit antiserum	2	0	0		185	54	0	0	27	9	0	0	2	0	0	0	1	0	0	0
	(*b*) " 2 " "	∞	2000	67		∞	1000	250	49	∞	1000	150	44	700	90	6	31	500	146	32	7
	(*c*) " 13 " "	∞	2000	235		∞	1000	3 0	7	∞	1000	150	18	500	100	10	35	200	58	10	3
	(*d*) Normal rabbit serum	∞	2000	209		∞	1000	500	33	∞	2000	150	200	700	200	71	52	500	106	35	3
	(*e*) Type 48 " antiserum	∞	1000	282																	

* See Table I and Methods for technique of indirect bactericidal test. The direct test (Table III(*a*)) is carried out in the same way as the indirect except that no serum or plasma is added.

‡ Strain B574 was obtained from a throat culture of LA during the course of an untreated pharyngitis.

sults shown in Table III, the bactericidal effect of the type 28 rabbit antiserum was tested against strains of a variety of other types and also against group A strains of unknown type.

Each heterologous strain of known type was tested with its homologous type unabsorbed antiserum, as well as with unabsorbed type 28 rabbit serum and normal rabbit serum. Growth of each strain was strongly inhibited by homologous type immune serum. The types tested were: types 1, 2, 3, 6, 8, 9, 12, 13, 18, 25, 26, 28, 30, 36, 38, 40, 41, 43, H105, and Red Lake, as well as type 48 described below. The unabsorbed type 28 rabbit serum did not inhibit any of the heterologous strains, but strongly inhibited the homologous type 28 strain, T28/150A. Type 28 antiserum specifically absorbed, so that it contained only type 28 M antibody, inhibited the growth of type 28 strains in the same way.

These tests established the specificity of the rabbit antiserum.

The specificity of the postinfection human blood, LA, was tested in direct bactericidal tests with 18 strains of 11 different serological types and with 23 group A strains for which the type could not be determined. These tests were all negative although, like the immune rabbit serum, the human blood containing type 28 antibody inhibited all the type 28 strains strongly.[5]

Precipitin Reactions.— M-precipitin reactions of the 5 strains identified as type 28 by means of the bactericidal test were reinvestigated. The results in earlier tests had been equivocal, but the serum prepared by immunization with the second-passage series strain, T28/150A, gave good reactions.

After absorption with an R-containing strain of known heterologous type and with the usual strains employed in absorbing antisera to remove non-type-specific antibodies, the serum was free of cross-reactions and gave type-specific M precipitin reactions with the 5 strains listed in Table III. It was negative with other R-containing strains, as well as with representatives of the other known serological types. With the second mouse-passage series of strain T28 and with strain C294 after 154 mouse passages, stronger reactions were obtained than with strains which had not been passed through mice. The variants of strain T28 reflected, in the strength of their M precipitin reactions, the varying amounts of M antigen indicated by the bactericidal tests. Serum absorbed for these precipitin tests also gave type-specific inhibition of growth of type 28 strains in bactericidal tests.

It was thought important to determine whether the variant derived from strain T28 by a long series of mouse passages could be identified as a derivative of the original strain.

Antisera prepared with the original strain were too weak in type-specific antibody to be used, either in precipitin or in bactericidal tests. However, the antisera prepared with the second passage series strain not only gave type-specific precipitin reactions with the original strain before mouse passage, though not of as high titer as the derived variant, but also inhibited the growth of the original strain in bactericidal tests.

Table II shows the strong inhibitory action of antiserum made by immunization

[5] This individual also had type 6 antibodies, but these are not effective against type 28 strains.

with the passage strain when used in bactericidal tests with the original strain T28, and with the second mouse-passage series strain, T28/150A, thus establishing the relationship of the two strains.

Antisera prepared with the first mouse-passage series strain (the 51st passage), when the strain was so much degraded that it would not grow in normal human blood in bactericidal tests, also inhibited to some extent the growth of the original strain T28, and the second series passage strain T28/150A.

In order to test the effect of tryptic digestion on type 28 M antigen, a hydrochloric acid extract was prepared from the second mouse-passage strain T28/150A. The extract contained both R antigen and type 28 M antigen. This solution was treated with crystalline trypsin, after which it no longer gave a precipitin reaction with the absorbed type 28 anti-M serum although it still reacted well with anti-R serum. The results with the type 28 extract are consistent with previous findings that type-specific M antigens are digested by trypsin but that R antigen is unaffected by tryptic digestion although it is destroyed by pepsin.

Slide Agglutination Tests.—Certain of the antigen-antibody reactions of group A streptococci can also be investigated by the technique of slide agglutination. Three of the serologically active proteins studied in group A streptococci give rise to antibodies which may agglutinate the streptococci containing them. These are the type-specific M antigens, the T antigens which may be present in strains with different M antigens, and the R antigen which occurs in some, but not all, of the strains of the three serological types described in this report. Anti-R and anti-M agglutinins are found in specifically absorbed sera of rabbits immunized with type 28 strains, but no anti-T agglutinins have been encountered so far. Correspondingly, members of this type are not agglutinated by any of the known anti-T antibodies. It is concluded, therefore, that these 5 type 28 strains probably do not contain T antigen.

The demonstration of type 28 M antigen in these strains by means of the agglutination reaction was accomplished by absorbing type 28 antiserum with an R-containing strain of another serological type, for example, with strain C649A which is a member of type 2, or with strain C510, which belongs in type 48. When the serum was completely absorbed with respect to R antibodies, as shown by negative precipitin reactions, it no longer agglutinated heterologous R-containing strains but showed type-specific agglutination of the 5 strains included in type 28.

By agglutination, therefore, as well as by bactericidal and M precipitin reactions, the evidence indicates that these 5 strains are members of a single serological type, designated as type 28 and represented by strain Small (Griffith) as the reference strain for the type.[6]

Identification of Members of Type 48.—Thirty-eight R-containing strains and 2 without R antigen were found to belong to a new type designated as type 48.

The strains were obtained during the last 15 years from many parts of this country and from Canada and England. At first they were thought to belong in type 13 on the basis of precipitin reactions with type 13 absorbed anti-M sera. Many of these reactions were slight,

[6] Strain Small is designated as T28 in this laboratory.

but they were often strong enough to raise the question of their significance. The decision was difficult to make on the basis of the precipitin reactions because type 13 antisera are not always strong and may also be hard to absorb free of antibodies giving non-specific cross-reactions with occasional strains.

Most of these type 48 strains contain R antigen, and all of them contain a different T antigen from the one usually found in type 13 streptococci. In slide agglutination tests with absorbed antisera, these strains were agglutinated by the T antibodies, common to types 4, 24, 26, 29, and 46. The T antigen responsible for this reaction is usually referred to as the 4-24 T antigen. Ten type 13 strains, none of which contain R antigen, were studied for comparison. None of them have the 4-24 T antigen, but all have the T antigen usual for type 13 strains and usually common to types 3, 13, and B3264.

Bactericidal tests made with type 13 antisera and 8 of the type 48 strains, as well as 6 type 13 strains, showed a considerable degree of inhibition of growth. None were as strongly inhibited as the mouse-passage strain, T13/51, with which the rabbits were immunized in the preparation of the type 13 antisera.

Potent antisera against type 48 strains, when finally obtained, showed that type 48 strains give specific M precipitin and bactericidal reactions in both type 48 and type 13 antisera, but that type 13 strains react only in type 13 antisera. The reactions of the type 48 strains are usually less strong in type 13 antisera than in type 48 antisera. Furthermore, strain variation is evident in the bactericidal reaction of type 48 strains when tested with type 13 antisera.

The type 48 antisera, however, gave convincing evidence of a one-sided relationship between types 13 and 48. This was shown not only in the M precipitin reactions but also in the specific, strongly positive bactericidal reactions of type 48 antisera with type 48 strains and the lack of such reactions with type 13 strains. Examples of these relationships are shown in Table IV. Fifteen type 48 strains and 6 type 13 strains were used repeatedly in bactericidal tests with several different antisera for each type, and gave essentially similar results, if allowance is made for the fact that not all strains are as suitable for bactericidal tests as those recorded in the table. Bactericidal tests were negative with type 48 antisera and heterologous strains of other types tested: types 2 (including R-containing strains), 24, 26, 28, 29, 46, as well as a number of strains of unknown types.

M-precipitin reactions were performed repeatedly with all 40 of the type 48 strains and with all available type 13 strains. Typical examples of these reactions with the stock absorbed anti-M sera for types 13 and 48 are recorded in Table V. These antisera gave no cross-precipitin reactions with R-containing strains of types 2 and 28, nor with other heterologous type strains. Reciprocal absorption experiments to test the ability of types 13 and 48 strains to remove anti-M precipitins confirmed the one-way immunological relationship of these strains. In each case, the homologous type strains completely absorbed the anti-M precipitins after one or two absorptions with heat-killed bacteria. In addition, the type 48 strains also absorbed completely from type 13 antiserum the anti-M antibodies reactive with both types 13 and 48 strains. On the other hand, the type 13 strain did not absorb the anti-M precipitins from type 48 antiserum even after three absorptions. These results are shown in Table V.

The results of bactericidal tests with numerous reciprocally absorbed antisera were less determinate than the M precipitin reactions because the margin was slight between specific absorption with types 13 and 48 strains and absorption with heterologous type strains. More potent antisera would be necessary for satisfactory testing of reciprocal absorption by means of bactericidal tests.

The two type 48 strains which did not possess R antigen gave type-specific reactions indistinguishable from the R-containing strains of type 48. One of these strains,

TABLE IV

One-Way Cross-Relationship between Types 13 and 48
Bactericidal Tests

Representative strains tested		Results of bactericidal tests No. of colonies				
Type 13	No. of streptococci inoculated	*Inoculum*				
		1000	124	20	9	2
Strain T13/51	No. of streptococci at end of test with	*Bactericidal tests*				
	(*a*) Normal rabbit serum	∞	4000	4000	500	39
	(*b*) Type 13 antiserum	12	0	0	0	0
	(*c*) " 48 "	∞	4000	500	152	16
Type 48	No. of streptococci inoculated	*Inoculum*				
		174	65	17	7	
Strain C510/100b	No. of streptococci at end of test with	*Bactericidal tests*				
	(*a*) Normal rabbit serum	2000	1000	115	107	
	(*b*) Type 13 antiserum	339	193	30	7	
	(*c*) " 48 "	120	18	0	0	

* See Table I and Methods for techniques. Normal human blood was present in all these bactericidal tests.

B403, has been useful in the immunization of rabbits for type-specific anti-M sera because no R antibody was produced. It also seemed to be a better immunizing strain than several of the R-containing strains which were tested.

Identification of Sixteen R-Containing Strains as Members of Type 2.—The remaining sixteen strains containing R antigen gave positive precipitin reactions with absorbed type-specific type 2 antiserum.

Four of these cultures were obtained in 1942–43 either from Boston or from the Newport Naval Training Station; 12 strains were isolated in Rochester or Syracuse within the last few years. These cultures were compared with 12 previously identified strains of type 2 which did not give positive precipitin reactions with potent anti-R sera and, therefore, did not contain R antigen. The identification of these cultures by means of M precipitin reactions was not entirely satisfactory, partly on account of the rapid loss of M antigen by type 2 strains and partly on account of cross-reactions which tended to occur even with normal rabbit sera.

Since all known type 2 strains contain T antigen also, this additional component was investigated as a further means of identification. The T antigen in the hands of many workers has proved a stable, as well as specific, antigenic component of type 2 streptococci (25). Agglutination reactions dependent upon type 2 T antigen and the corresponding antibody are useful in the identification of type 2 strains. Slide agglutination reactions with absorbed type 2 antisera containing anti-T antibodies were uniformly strong and specific with all of these strains, irrespective of whether they contained R antigen.

It was noted by Griffith that the serum of rabbits immunized with an R-containing group C streptococcus, type 21 (Griffith's strain Radford), agglutinated group A

TABLE V

Reciprocal Absorption Experiment, Types 13 and 48 M-Precipitin Reactions

Antisera	Results of M precipitin tests with streptococcal extracts	
	Type 13 extract	Type 48 extract
Type 13 anti-M serum*		
(*a*) Untreated	++	++
(*b*) Absorbed once with type 13 streptococci	−	−
(*c*) Absorbed once with type 48 streptococci	−	−
Type 48 anti-M serum*		
(*a*) Untreated	−	+++
(*b*) Absorbed three times with type 13 streptococci	−	++
(*c*) Absorbed three times with type 48 streptococci	−	−

* The stock antisera were previously absorbed with strains other than types 13 and 48 for use as anti-M sera for precipitin tests.

Precipitin reactions recorded on a scale of − to ++++.

streptococci, type 2 as well as the homologous group C culture (22). The reactions appear to be due to the T–anti-T antigen antibody system. All the type 2 strains included in this report were specifically agglutinated by a group C, type 21 antiserum which had been specifically absorbed so as to remove anti-R antibodies. An antiserum prepared with an R-containing group C strain other than type 21 failed to agglutinate type 2 strains unless they contained R antigen. This indicates that R antibodies do not play a part in the specific cross-reactions between group A, type 2 and group C, type 21.

The bactericidal test was applied to a number of these strains for additional confirmation of type 2 specificity. Satisfactory reactions were obtained with the 11 R-containing group A strains tested and with 9 of the 10 type 2 strains which did not contain R antigen. Representative examples of these tests are shown in Table VI. The growth of the strain (T2/44/Rb 4) with which the serum was prepared was more strongly inhibited than that of other strains (exemplified by strain C649A) although the latter tests are considered satisfactory. The superior results with the

homologous strain may be a reflection of the increased content of M antigen of this strain, resulting from serial passages in mice and rabbits and leading to increased absorption of anti-M antibody, or to other differences of unknown nature which may affect the quantitative relationships of this complex biological test.

TABLE VI

Relationship of R-Containing Type 2 Strains to Those without R Antigen

Strains and sera tested		Results of bactericidal tests No. of colonies			
	Untreated sera				
Type 2 strain with R antigen (Strain C649 A)	No. of streptococci inoculated	*Inoculum*			
		45	15	2	0
	*No. of streptococci at end of test with	*Bactericidal tests*			
	(*a*) Normal rabbit serum	1000	143	34	72
	(*b*) Antiserum to strain T2	242	38	0	0
	(*c*) " " " C649A	35	8	0	0
	Untreated sera				
Type 2 strain without R antigen (Strain T2)	No. of streptococci inoculated	*Inoculum*			
		86	16	1	
	No. of streptococci at end of test with	*Bactericidal tests*			
	(*a*) Normal rabbit serum	1500	1000	484	
	(*b*) Antiserum to strain T2	0	0	0	
	(*c*) " " " C649A	60	80	0	
	Absorbed sera				
Strain T2 (Same as above)	No. of streptococci inoculated	*Inoculum*			
		87	25	7	
	No. of streptococci at end of test with	*Bactericidal tests*			
	(*a*) Normal rabbit serum	592	576	81	
	(*b*) Type 2 antiserum: untreated	9	0	0	
	(*c*) " " " : absorbed once with				
	1. Type 2 strain, T2 (without R)	271	8	57	
	2. " 2 " C649A (with R)	700	178	0	
	3. " 13 " T13/88 (without R)	0	0	0	
	4. " 28 " T28/150A (with R)	0	0	0	
	5. " 48 " C510/71 (with R)	0	0	0	

*Normal human blood was present in all these bactericidal tests. See Table I and Methods.

Strain T2 had 44 passages through mice, 4 through rabbits, then 91 through mice.

Strain C649A had 134 passages through mice.

Further evidence that R-containing type 2 strains are essentially the same as type 2 strains devoid of this antigen was furnished by specific absorption experiments. Aliquots of a type 2 antiserum were absorbed respectively with R-containing strains of the three types studied, types 2, 28, and 48, and with strains of types 2 and 13

which lacked R antigen. These samples of absorbed sera, together with suitable untreated and normal control sera were employed in a bactericidal test for ability to inhibit the growth of the standard type 2 strain, T2. The data in the bottom section of Table VI show that the R-containing type 2 strain absorbed the specific antibody from the serum in the same manner as strain T2 which had no R antigen. With the other absorbing strains, the results of the bactericidal tests were the same as those with the untreated serum.

TABLE VII

Various Combinations of Antigens Found in R-Containing Streptococci and Closely Related Strains

Antigenic components				Serological classification	Remarks
Group antigen (polysaccharide)	R antigen (protein)	M antigen (type-specific protein)	T antigen (protein)		
A	—	2	2	Group A, type 2	Type 2 T antigen also occurs in some group C, type 21 strains and in some group G strains (see below)
A	R	2	2	" " " 2	
A	R	28	—	" " " 28	Griffith's Strain Small is the reference strain (T 28)
A	—	I3	3-13-B3264	" " " 13	Type 13 M antigen does not cross with type 48 serum.
A	—	48	4-24-26-29-46-48	" " " 48	Type 48 M antigen crosses with type 13 serum.
A	R	48	4-24-26-29-46-48	" " " 48	
C	R	—	2	Group C, Griffith's type 21	Not known whether some type 21 strains lack R antigen.
C	R	—	—	Group C, not type 21	
C*	—	—	—	Group C, types not known	
B‡	R	—	—	Group B, type III	R antigen occurs in several, but not all, group B, type III strains.
G§	R	—	2	Group G, types not known	R antigen occurs in a few group G strains.

* Many "large colony" (26) group C strains have R antigens with serological specificities different from the R antigen studied here (unpublished data).

‡ Type specificity is usually based upon type-specific polysaccharides (S antigens) in group B (27, 28).

§ See reports of Crowley (29) and of Maxted (25, 30) for indications that some group G strains contain type 2 T antigen and that some may contain R antigen.

Although the data so far presented indicate strongly that R-containing type 2 strains are, except for this antigen, serologically identical with other type 2 strains, the reciprocal of the absorption experiment given above has not been carried out since serum for this purpose is not at present available.

Strain C649A/120 was used as a representative strain to test the possible bactericidal effect of potent antisera of other types on R-containing members of type 2. No significant effect on this R-containing type 2 strain was observed with the type-specific antisera tested: these included types 3, 12, 18, 25, 28, 36, 40, 41, 43. The only cross-reactions found were with types 13 and 48 antisera.

A similar cross-reaction between two R-containing strains, now shown to belong in types 2 and 48, was studied previously by means of mouse protection tests; marked cross-protection was found with the antisera available at that time (10). Absorption of each serum with a suspension of the other streptococcus resulted in removal of cross-protective antibodies without affecting the protective value of the serum for its homologous strain. Cross-reactions between types 2, 13, and 48 have sometimes been observed in bactericidal tests. They are somewhat erratic in occurrence, and are not usually as strong as the homologous reactions. They tend to be removed by reciprocal cross-absorption without affecting the homologous bactericidal reaction. Corresponding precipitin reactions have not been seen. The antigen responsible for the cross-reaction was not identified in the earlier mouse protection experiments nor in the present experiments with the bactericidal test.

The relationships and distribution of the various antigens occurring in different combinations in R-containing or related streptococci have been summarized in Table VII. The R antigen occurs in certain strains of groups A, B, C, and G. In group A it has been found in some strains of types 2, 28, and 48. The M antigens by which these types are defined may, therefore, occur either with or without R antigen.

The type 2 T antigen occurs in all known group A, type 2 strains and in some strains of groups C and G. It stimulates potent antibody production in rabbits. Marked agglutination reactions are caused by this antigen-antibody system, and the corresponding precipitin reactions have also been reported (25). The type 2 T antigen in these diverse strains appears to be antigenically the same.

The T antigens of the other types concerned are indicated and have been studied previously (15, 31). In type 28 no T antigen has been found; types 13 and 48 have distinct T antigens, in each case shared with serological types not pertinent to the present study.

DISCUSSION

Attempts to classify R-containing group A streptococci into serological types in the usual way by means of M precipitin reactions have frequently given equivocal results. Many strains classified as types 2 and 13 when first isolated tend to lose their capacity to produce M antigen. Rabbits immunized with such cultures yield antisera with only low titers of type-specific anti-M antibodies. The results are complicated in the case of R-containing strains by high titers of non-type-specific anti-R antibodies.

In earlier work passive protection tests in mice were employed for a partial

analysis of protective antibodies against these strains. Because of difficulties encountered in obtaining mouse virulent strains and satisfactory protective antisera, the indirect bactericidal test was substituted for mouse protection tests in this study, and the R-containing strains were classified into 3 serological types.

The bactericidal test is thought to reflect the same antigen-antibody reaction as the mouse protection test, and is a sensitive means of identifying either M antigen or its antibody. This test has also been used satisfactorily for the same purpose by Maxted (11). The ability of streptococci to grow in normal human blood, which is a requisite condition of this test, appears to be dependent upon the amount of M antigen produced by the culture under examination. Increased M production frequently found in strains subjected to animal passage results in a proportionately increased capacity to grow in human blood. Glossy strains with little or no M antigen are unsuitable for use since they grow poorly in human blood under the conditions of the test.

The type-specific inhibition of growth demonstrable in bactericidal tests was found to be correlated directly with the M antibody. R and T antibodies were without effect. In this test, as in mouse protection tests, unabsorbed sera may, therefore, be used.

The union of M antibody with the M antigen on the surface of the bacterial cell increases the susceptibility to phagocytosis by human leukocytes. In the bactericidal test the inhibitory effect of antibody is most marked with strains containing the largest amount of M antigen. Conversely, it has been found that with glossy strains in which the concentration of M antigen is low, the effect of adding type-specific antibodies is slight. This suggests that since glossy organisms do not absorb anti-M antibody the human leukocytes become engorged with easily phagocyted glossy streptococci as readily in the control series as they do in the test series, even though a few organisms coated with anti-M antibody may also be present in the latter.

With cultures yielding intermediate amounts of M antigen, yet sufficient to allow satisfactory growth in human blood, the bacterial population may be composed of a mixture of variants with and without M antigen in which the proportion containing M antigen in normal amount is higher than in the glossy strain. If the leukocytes ingest streptococci without M antigen as readily as M-containing variants coated with type-specific antibody, the differential between the control tubes and those containing antiserum would be less than in virulent cultures composed almost entirely of M-containing organisms.

An alternate hypothesis might be that incomplete phagocytosis occurs with intermediate strains because antibody does not adhere well to the bacteria owing to a low concentration of M antigen on all of the individual cocci. The resolution of this problem is hampered by the lack of a quantitative method of determining the amount of M antigen produced.

Attempts were made to substitute rabbit for human leukocytes in bactericidal tests. Except for an occasional individual rabbit, the results were unsatisfactory. This was true even in direct bactericidal tests using the whole blood of immunized rabbits with demonstrably high anti-M antibody titer. On the other hand, with human blood, in which the anti-M antibody content was low, as indicated by inability to demonstrate precipitins and by poor results in passive mouse protection tests, excellent inhibition of growth was obtained in direct bactericidal tests with whole blood. The comparatively low concentration of anti-M antibody in human serum as compared to the high type-specific antibody titer in rabbit sera can be shown in the indirect test by using increasing dilutions of serum.

Whether or not the difference in the ability of human and rabbit leukocytes to inhibit growth of group A streptococci in the presence of anti-M antibodies is in any way related to the fact that naturally occurring infections with these organisms do not occur in rabbits, is at the present time not understood.

SUMMARY AND CONCLUSIONS

In further study of streptococci having the R antigen, the bactericidal test has been used instead of the mouse protection test in investigating the type-specific M antigens of these organisms. The results have been confirmed by M anti-M precipitin tests, and a correlation between the M and T antigens of the strains has been shown.

On the basis of a specific M antigen, type 28 has been shown to comprise Griffith's strain Small and four other R-containing strains.

A number of other strains previously thought to belong to type 28 on the basis of R antigen reactions have now been identified as belonging either to type 2 or to a new type, designated 48, which shows a one-way cross-relationship to type 13.

The bactericidal test is suggested as a useful method for assessing M antigen in group A streptococci and for establishing type-specificity by means of a biological test which is more widely applicable than the standard mouse protection test.

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