THE INDIRECT BACTERICIDAL TEST AS A MEANS OF IDENTIFYING ANTIBODY TO THE M ANTIGEN OF *STREPTOCOCCUS PYOGENES*

W. R. MAXTED

From the Streptococcus Reference Laboratory, Central Public Health Laboratory, Colindale, London, N.W.9

Received for publication June 1, 1956

THE accepted method for the identification of serotypes of *Streptococcus pyogenes* (Lancefield Group A streptococci) is based on a precipitin reaction with antisera against the type-specific M antigen. The criteria for an anti-M serum are that it should give precipitation with the acid-extracted antigen of the vaccine strain, and of other strains of the same " type ", and should not react with such extracts after they have been treated with trypsin; and that it should confer passive protection on mice when given twenty-four hours before a challenge dose of the organism.

Many workers who have had experience of the preparation of type-specific precipitating sera have come across non-specific trypsin-sensitive antigens which might be confused with M substance. It is also a common experience to find that certain type sera are always difficult to make; such types as 8, 11, 13, 22, 25, and 27 have never given satisfactory precipitating sera (Williams and Maxted, 1953). Highly mouse-passaged variants of these types sometimes produce antisera which, after absorption, precipitate well with extracts of the vaccine strain but not with extracts of the same strain before mouse passage.

When the nature of the antibody is in doubt, the ability to confer passive protection on mice is the most reliable method of establishing whether it is an M antibody. However, because of the low virulence of so many of the vaccine strains it is often impossible to carry out the mouse protection test. This is often so even though the strains have been freshly isolated from cases of acute streptococcal infection in man.

The bactericidal test has been used to show the development of protective antibody in the serum of patients convalescent from streptococcal infection. The direct bactericidal test uses the patient's whole blood (Todd, 1927) and the indirect test makes use of the blood from normal children, to which a small amount of patient's serum is added (Rothbard, 1945). The M antibodies thus introduced confer bactericidal powers upon the blood against streptococci of the same M type. It seemed possible that such a test might well be a substitute for mouse protection in the testing of anti-M rabbit sera. The main points of interest were to identify M antibody reacting with avirulent strains, to use the same technique to identify M antibody made from possible new types of streptococci, and to

study the reactions of strains having a trypsin-labile antigen which gave only moderate and possibly non-specific precipitation with type antisera.

MATERIALS AND METHODS

Strains

These were of three classes.

Class I.—Strains used to make some of the accepted anti-M sera which have always been easy to prepare. These strains have been subdivided into two classes, I*a* virulent for mice and I*b* non-virulent.

Class I*a*	Type	1	R154	Type	30	D24/46
		17	J17E/19		36	C119
		19	J17D/50		39	C95
Class I*b*	Type	5	T5B	Type	9	41445/10

Class II.—Recognised type strains, the M antisera of which have nearly always proved difficult to prepare, and about which there has often been doubt as to the true nature of the reaction obtained.

Type	2	T2/44/Rb5	Type	8	SF4matt
	4	R54/2692		22	45128

All these were avirulent for mice.

Class III.—Provisional new types, the antisera to which, though reacting with extracts of the homologous strains, also reacted with trypsin-labile antigens extracted from cells of other types.

Provisional types : " Lily ", " Angas " and " Corby ".

Antisera

The sera were prepared in this laboratory by the usual method : rabbits were injected with heat-killed suspensions of washed cells on three successive days in each week.

Blood

Fresh normal human adult blood was used. The donors were selected because their blood allowed the multiplication of the particular strains. All blood was heparinised (8 units/ml.).

Blood from normal Rhesus monkeys was used in some experiments.

Bactericidal test

Corked sterile tubes 2 in. × ½ in. (5·1 × 1·3 cm.) were set up in Wassermann racks and 0·3 ml. of heparinised blood was run into each followed by 0·02 ml. of rabbit anti-M serum which was added to each tube with a dropping pipette. Normal rabbit serum or saline was added to a control set. Overnight cultures of the streptococcus were diluted in 10 per cent broth saline and 0·02 ml. of diluted culture was added to the appropriate mixture. The usual test range dilution of culture was 10^{-5}, 10^{-4}, 10^{-3}. The number of viable streptococcal units in 0·02 ml. of highest dilution averaged between 50 to 100. The mixtures were put into a Kahn shaker set to shake for 3 min. every 15 min. with a time clock. The wide rounded base of the tubes allowed adequate mixing.

The mixtures were explanted at 3 hr. and 20 hr. A large standard loop was filled with blood mixture and spread evenly over ⅛th sections of duplicate blood agar plates which were incubated overnight and the growth recorded over the range − to ++++.

RESULTS

Glossy variants of streptococci failed to grow in normal blood while the matt M-containing variants usually grew readily. It was a fair assumption that strains

capable of growing contained M antigen, and in all cases where well-established type strains were used and grew freely, this was so.

The use of rabbit serum in human blood mixture

Normal human blood was tested against class I*a* M type strains ; blood that allowed all the type strains being investigated to grow was selected. Each strain was set up against mixtures containing normal rabbit serum, the homologous antiserum and at least one heterologous antiserum.

Explants were made at 3 hr. and 20 hr. The latter period of incubation provided a rather severe test, but it was found that with good anti-M sera the blood was usually able to kill the organisms completely or to restrict their growth to a minimum. The results using three known types, 17, 18, and 30, are shown in Table I.

Such mixtures of serum and normal human blood were highly bactericidal and the results were reproducible. Other good anti-M rabbit sera (Types 24, 36, and 39) were used in the same way and proved equally satisfactory.

Blood from other species

The necessary search for a suitable normal human blood is tiresome. Rabbit blood, even from rabbits that provided excellent M antiserum, had proved to have no bactericidal action whatever, even for the streptococcus with which they had been inoculated. Monkey blood was therefore tested.

Heparinised blood obtained by heart puncture of the monkeys was supplied by the Virus Reference Laboratory, and was used in exactly the same way as in the successful tests with human blood. The results with the first two animals are shown in Table II ; clearly the blood from these was quite suitable. Of the next two animals used one yielded satisfactory blood while that of the other behaved in the same way as rabbit blood. No increase in killing power was obtained by adding anti-M serum. However it seems possible to obtain a satisfactory animal.

*Avirulent M strains and their antisera (Class I*b)

The strains used in the experiment reported in Tables I and II were either mouse-virulent or could easily be made so and the sera were all well established anti-M sera. These strains and sera could therefore have been tested just as well by mouse protection tests. This was not the case with strains of Class I*b* represented here by type 5 and type 9 (Table III).

Acid extracts of type 5 reacted well with the antiserum. The antiserum made against type 9 gave moderate precipitation with the homologous extract but did not react with all type 9 strains encountered. Both strains were avirulent for mice but were able to multiply when inoculated into suitable human blood. The indirect bactericidal test showed that antisera made from both strains contained a phagocytosis-enhancing antibody but that the type 9 antiserum was rather weak in this respect.

The usefulness of the test using non-virulent strains has been confirmed with other well established types, *e.g.*, type 1, type 6 and type 29.

Avirulent strains producing doubtful M antisera (Class II)

The strains and sera in this series were of types 2, 4, 8, and 22 (Table IV). The type 2 serum gave good precipitation, but not as many freshly isolated type 2 strains identified by agglutination reacted with it as were expected.

The type 2 serum R434, tested against two different type 2 strains, had a bactericidal effect and this indication that it contained M antibody confirmed earlier work with this serum (Maxted 1953). Type 4 was of particular interest since the absorbed serum precipitated moderately well with most strains identified as type 4 by agglutination. Extracts of these strains however often reacted with sera of related types such as 29 and 46, which would not be expected with a true M antigen. Not many type 4 strains grew well in human blood, but when strains that grew freely were used in an indirect bactericidal test with the antiserum, there was no evidence of enhanced phagocytosis, which suggested that the precipitation reaction was not due to type 4 M antibody. This has been confirmed by other work done in this department (Hambly, in preparation).

The type 22 serum reacted with extracts of the vaccine strain only but the bactericidal test failed to show any enhanced killing power. Type 8 antiserum also failed to promote bactericidal action but this was expected since it gave poor and only non-specific precipitation with acid extracts of the streptococci. None of the strains in this series was virulent enough to be used as a challenging strain in protection tests in mice.

Lancefield and Perlmann (1952) have shown that the " type 28 " R antigen is quite unlike the typical M antigen : it is resistant to trypsin digestion and its antibody fails to give passive protection. Many strains have the R antigen but in only two strains has there been a suggestion that the more usual M-like antigen was also present. These were two strains which Lancefield and Perlmann had rendered highly mouse-virulent after numerous mouse passages. Antisera against the passaged strains protected mice against challenge with them.

In previous work here, attempts had been made to demonstrate a typical M antigen in stock strains of type 28 giving strong R-antigen reactions ; many sera were made, all of which contained much R antibody and some of which also gave precipitation with trypsin-sensitive protein fractions extracted from the streptococcus. However, bactericidal tests with these strains and their antisera showed no enhancement and so failed to confirm that the antibodies were the result of stimulation by M protein. Parallel tests could not be done in mice because the strains failed to increase in virulence even after numerous mouse passages.

New types giving reproducible and characteristic T agglutination (Class III)

Three strains known as " Lily ", " Angas " and " Corby " were recognised by their characteristic agglutination patterns in an epidemiological study in a children's home (Holmes and Williams, in preparation). They failed to precipitate with any type antisera. Attempts were therefore made to prepare specific sera for these three strains. One antiserum against " Lily " precipitated well with the homologous strain but also with a large variety of strains which, according to the agglutination pattern, were unrelated. The second serum, against " Angas ", was specific in its precipitation but no strains have so far been found outside the community under investigation. The serum against the third strain, " Corby ",

TABLE I.—*Inhibition of Growth of Streptococci in Human Blood to which Anti-M Rabbit Serum had been Added*

Addition to blood.	Culture dilution. Type 17. 10^{-5}.	Type 17. 10^{-4}.	Type 17. 10^{-3}.	Type 19. 10^{-5}.	Type 19. 10^{-4}.	Type 19. 10^{-3}.	Type 30. 10^{-5}.	Type 30. 10^{-4}.	Type 30. 10^{-3}
Saline . .	+/++++	+++/++++	++++/++++	+/+++	+++/++++	+++/++++	±/++++	++/++++	+++/++++
Type 17 antiserum	−/−	−/++	±/+++	−/++++	++/++++	+++/+++	±/++++	+/++++	+++/++++
,, 19 ,,	+/++++	++/++++	+++/++++	−/−	±/−	−/−	±/++++	+/++++	++/++++
,, 30 ,,	±/++++	++/++++	+++/++++	+/++++	++/++++	+++/++++	−/−	−/−	±/−

In this and the following Table : − = no growth ; ± . . . ++++ increasing amounts of growth. The symbol before the stroke refers to the sample taken after 3 hr. incubation, that after the stroke to the sample taken after 20 hr.

TABLE II.—*Inhibition of Growth of Streptococci in Monkey Blood to which Anti-M Rabbit Serum had been Added*

Addition to blood.	Culture dilution. Type 17. 10^{-5}.	Type 17. 10^{-3}.	Type 19. 10^{-5}.	Type 19. 10^{-3}	Type 30. 10^{-5}.	Type 30. 10^{-3}.
Monkey No. 11						
Saline . . .	+/++++	+++/++++	±/++++	+++/++++	±/++++	+++/++++
Type 17 antiserum	−/−	±/±	+/++++	+++/++++	+/++++	++++/++++
,, 19 ,,	+/++++	+++/+++	±/−	±/++	+/++++	+++/++++
,, 30 ,,	+/++++	+++/++++	+/++++	+++/++++	−/−	±/±
Monkey No. 12						
Saline . . .	+/++++	+++/++++	±/++++	+++/++++	++/++++	+++/++++
Type 17 antiserum	−/±	±/±	±/++++	+++/++++	N.D.	N.D.
,, 19 ,,	+/++++	+++/++++	−/−	±/−	N.D.	N.D.
,, 30 ,,	++/++++	++++/++++	+/++++	+++/++++	−/−	±/−

N.D. = not done.

TABLE III.—*Bactericidal Test of Non-virulent Strains with their Type Sera Containing Known M Antibody*

Addition to blood.	Culture dilution. Type 5. 10^{-5}.	Type 5. 10^{-4}.	Type 9. 10^{-5}.	Type 9. 10^{-4}.
Normal rabbit serum	+/++++	+++/++++	+/++++	++/++++
Type 5 antiserum	−/+	−/+	N.D.	N.D.
„ 9 „	N.D.	N.D.	+/−	+/++
Heterologous type serum	+/++++	+++/++++	±/+++	+++/++++

TABLE IV.—*Bactericidal Test of Non-virulent Strains with their Antisera, which Contain Doubtful M Antibodies*

Addition to blood.	Culture dilution. Type 2. 10^{-5}.	Type 2. 10^{-3}.	Type 4. 10^{-5}.	Type 4. 10^{-3}.	Type 8. 10^{-5}.	Type 8. 10^{-3}.	Type 22. 10^{-5}.	Type 22. 10^{-3}.
Normal rabbit serum	±/++++	+++/++++	+/++++	++/++++	+/+	++/++++	±/++++	++/++++
Homologous type serum	−/±	−/±	±/++++	++/++++	+/+	++/++++	−/++++	+/++++
Heterologous type serum	±/++++	++/++++	±/++++	++/++++	++/++++	+++/++++	+/++++	+++/++++

TABLE V.—*Serological Reactions and Bactericidal Test of Provisional New Types with their Antisera*

Addition to blood.	Lily. 10^{-5}.	Lily. 10^{-3}.	Angas. 10^{-5}.	Angas. 10^{-3}.	Corby. 10^{-5}.	Corby. 10^{-3}.
Normal rabbit serum	+/+	++/+++	+/+	++/+++	++/++++	+++/++++
Homologous serum	+/−	+++/+++	−/−	±/−	−/−	−/−
Heterologous serum	++/++	+++/+++	+/++	+++/+++	+/+++	+++/++++
Range of precipitin reactions of serum	2/3/9/11/44/Lily		Angas		Corby	
Typical agglutination pattern of strain	3/13/B3264/12/27/Lily		11/27/44/Angas		5/27/44/Corby	

In the bactericidal test unabsorbed sera were used. For the agglutination and precipitation tests the sera were absorbed to remove non-type specific antibody.

precipitated well with the homologous type but was also prone to show cross reactions with some heterologous types.

Bactericidal tests (Table V) suggest that " Angas " and " Corby " are in fact new types, but the serum of strain " Lily " was as unconvincing in bactericidal tests as in precipitation with acid extracts. It seems unlikely that precipitates obtained with this strain are indeed due to M antigen antibody reaction. The strains were all avirulent for mice.

DISCUSSION

The bactericidal test can be used as an alternative to protection tests with mice for detecting M antibody. Many strains that cannot be used in mouse protection tests are usable in the bactericidal test. Although not all strains that contain M antigen will grow freely in human blood apparently free from specific antibodies, it is generally easier to find a suitable strain and blood than to raise the virulence of a strain by a long series of passages through mice.

It is interesting to find that Rhesus monkey blood may be used for testing Group A organisms. This seems to be the only example of an animal other than man that is suitable, and it is the only animal in which natural carriage of Group A streptococci has been observed.

In view of the consistent results obtained with strains known to give good M antisera, the failure of some types (4, 8 and 22) to produce bactericidal antisera confirms our suspicions that the precipitation results obtained with these sera are not due to M antigen.

In our hands stock strains of type 28 have failed to show any trace of M antigen in spite of yielding precipitinogens which can, like true M antigen, be destroyed by proteinase digestion. However these tests do not rule out the possibility that a true M antigen may be present in these strains.

The bactericidal test enables a large number of strains to be tested against many sera and there is no doubt that by its use some provisional M-types may well be discarded and equally certain that some, whose antisera give bactericidal action, will repay the additional investigations these results may stimulate. It is evident that the possession of M antigen alone is not enough to enable a strain to multiply in all human blood apparently devoid of antibody, and experience gained by the extended use of the bactericidal test may focus attention on other streptococcal fractions which play a part in virulence.

It is the custom to pass streptococci through mice to promote their virulence and increase their M content, yet many of these strains contain ample amounts of M antigen in their original form but only stimulate antibody production after mouse passage. The mouse passage, which is quite foreign to the streptococcus, may promote the development of a false precipitating antigen. Bactericidal tests, by representing a more normal environment, may give a more reliable index of the pathogenic abilities of some strains of *Str. pyogenes*.

SUMMARY

The ability of antiserum prepared in rabbits to enhance the bactericidal power of normal human blood for streptococci of the homologous type runs closely parallel with its ability to protect mice against intraperitoneal challenge. Monkey blood has also proved suitable for this purpose.

By this method it is possible to test many strains which are avirulent for mice yet contain ample M antigen, and also many sera which give M-like precipitations with acid extracts of the streptococcus but which probably contain a non-specific antibody.

REFERENCES

LANCEFIELD, REBECCA C. AND PERLMANN, GERTRUDE E.—(1952) *J. exp. Med.*, **96**, 83.
MAXTED, W. R.—(1953) *J. Path. Bact.*, **65**, 345.
ROTHBARD, S.—(1945) *J. exp. Med.*, **82**, 93.
TODD, E. W.—(1927) *Brit. J. exp. Path.*, **8**, 1.
WILLIAMS, R. E. O. AND MAXTED, W. R.—(1953) *Atti del Congresso Internationale di Microbiologia Roma*, **1**, 46.