STUDIES ON THE BINDING BETWEEN STREPTOCOCCAL M PROTEIN AND ANTIBODY*· ‡

By HOWARD M. GREY,§ M.D.

(From the Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh)

(Received for publication, October 3, 1961)

The type specific-M protein is associated with the pathogenicity of β-hemolytic streptococci (1–3). In addition, particular types of streptococci, and consequently particular M protein antigens, are associated with post streptococcal glomerulonephritis (4). Because of the apparent importance of this antigen in streptococcal infections and its non-suppurative complications, it was thought that investigation of the properties of the immune response to M protein might be of value in elucidating the pathogenesis of streptococcal disease.

None of the presently available tests of anti–M protein activity measure the initial antigen-antibody interaction, but rather they all depend upon secondarily related phenomena such as precipitation (5), agglutination (6), complement fixation (7), mouse protection (8), and opsinizing properties (9). These secondary phenomena are not necessary consequences of the primary antigen-antibody interaction, so that type-specific antibody from a given antiserum may escape detection if it is not capable of manifesting itself in the form of one or more of the secondary reactions mentioned above. In order to circumvent this difficulty and to better study the initial antigen-antibody interaction between M protein and its antibody, a test of primary binding capacity of antiserum has been employed. This test utilized as antigen a purified I^{131}-labeled type 12 acid extract which was soluble in 40 per cent saturated ammonium sulfate (I* M_{12}). Gamma globulin and globulin–I* M_{12} complexes are insoluble at this concentration of ammonium sulfate, so that it was possible to use the differential precipitation of radioactivity in the presence of type 12 immune serum as compared with normal serum and immune serum directed against other types of streptococci as a measure of the binding between I* M_{12} and its antibody. In addition, some information regarding the quality of this bond was obtained by measuring the dissociation of I* M_{12}-anti M_{12} complexes as a function of time.

* This investigation was supported in part by the National Institute of Allergy and Infectious Diseases, Grant E 1513.

‡ Supported in part by United States Public Health Service Training Grant 2E-1.

§ Present address: Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California.

Materials and Methods

Antigens.—

(*a*) *Heat-killed organisms:* Cultures of β-hemolytic streptococci[1] were stored at −20°C in tubes containing Difco Todd-Hewitt broth and 3 per cent whole rabbit blood. When ready for use, a tube was thawed and added to 4.5 liter quantities of Todd-Hewitt broth. Eighteen hour cultures were heated at 56°C for one hour and the cells harvested by centrifugation and kept at 4°C, merthiolate 1:10,000 was added as a preservative. Cells were used in this state for one month without detectable loss of M protein activity. The quantity of organisms used for each experiment was standardized turbidimetrically with a Bausch and Lomb colorimeter and was later correlated to the dry weight obtained from an aliquot of the same concentration of organism. The range of organisms used varied from 12 to 0.4 mg dry weight and will be indicated for each experiment.

(*b*) *I* M_{12} antigen:* A crude acid extract containing M protein was prepared from a type 12 β-hemolytic streptococcus according to the method described by Lancefield (5). The crude extract was fractionated twice with 60 per cent saturated ammonium sulfate (60 per cent SAS) at 4°C, and the resultant precipitate was brought into solution with isotonic saline and dialyzed free of ammonium sulfate. Ribonuclease[2] at a final concentration of 0.001 mg/ml was added to the fractionated extract, and this solution was allowed to incubate for 2 hours at 37°C and then was dialyzed at 4°C for 2 days in .01 M phosphate buffer pH 8.0 (10). Nitrogen determinations on the ribonuclease treated M_{12} extracts were performed by means of the micro-Kjeldahl method of Lanni *et al.* (11). One milliliter of this semi-purified M_{12} extract containing 420 γ N was labeled according to the method of Talmage *et al.* (12) using 35 mc I^{131} in the procedure. After dialysis to dispose of non-protein bound I^{131}, the solution was incubated overnight with a high titered antiserum prepared against several heterologous types of β-hemolytic streptococci. Saturated ammonium sulfate was then added to a final concentration of 40 per cent and after centrifugation the resultant precipitate was discarded. The latter procedure accomplished two purposes: (1) It cleared the antigen of that portion which was not soluble in 40 per cent saturated ammonium sulfate. (2) It removed most of the antigen molecules capable of binding with antibodies directed against heterologous types of streptococci. The iodinated antigen remaining in the supernate was then treated with one per cent trichloracetic acid, and after the precipitate was resuspended in and dialyzed against saline it was used as a test antigen. Antigen dilutions were carried out in one per cent normal rabbit serum (1:100 NRS) to a concentration of 0.002 to 0.0004 γ N/ml, the antigen concentration remaining constant for any given experiment.

Antiserum.—Albino New Zealand male rabbits weighing 2 to 3.5 kg were immunized with heat killed organisms, either intravenously according to the immunization schedule suggested by Lancefield (13) or subcutaneously with 1 ml of an emulsion consisting of 1 part arlacel, 9 parts bayol F, and 10 parts of a saline suspension containing 6 mg dry weight of heat-killed organisms/ml. In order to elicit a secondary response, a similar bacteria and adjuvant emulsion was injected one month after primary stimulation and twice monthly thereafter, the adjuvant injection being preceded on two successive days by subcutaneous injections of bacteria in saline in the same dosage as used in the adjuvant administration. Bleedings were carried out between 17 and 30 days after primary stimulation and 7 days after secondary stimulation with bacteria in adjuvant.

In the majority of experiments utilizing antiserum directed against heterologous streptococci, and unless otherwise noted, an antiserum directed against six heterologous types of group A streptococci[3] was used to test the type specificity of the antigen. This was prepared

[1] Kindly supplied by Doctors R. Lancefield, A. I. Braude and A. Stock.

[2] Worthington Biochemical Corporation, Freehold, New Jersey.

[3] Type 1, 2, 14, 17, 33, Red Lake.

by culturing each type separately and then pooling them prior to the immunization of animals. Antiserum directed against single strains of heterologous streptococci was also prepared.

All antisera were stored at −20°C without preservative until the time of use.

Absorption of Antisera.—Antisera were diluted 1:10 in borate buffer and absorbed 1 to 3 times with a volume of washed sedimented heterologous streptococcal cells equal to 1 to 2 times the volume of undiluted sera. Absorption was usually carried out with a mixture of two or more heterologous strains of organisms, although no difference in the efficiency of absorption of non-type-specific antibody was demonstrated when comparison between separate types and a mixture of types was made. Absorptions were carried out for 12 to 18 hours at 4°C. There was never any detectable M protein solubilization over this period of time.

Diluents.—Borate buffer (14) at pH 8.3, $\mu = 0.1$, was routinely used for all dilutions. Antigen was diluted in a 1:100 normal rabbit serum: borate buffer solution. This was done to provide sufficient protein carrier to prevent monolayering and denaturation of the antigen (15). All antisera were initially diluted 1:10 in borate buffer; the subsequent dilutions were made with 1:10 normal rabbit serum in borate buffer (1:10 NRS). The buffer served to control the pH of the reaction during the incubation of the antigen and the antibody, and when the antigen-antibody complexes were to be precipitated with ammonium sulfate, a final pH of 7.9 was maintained. The NRS diluent provided relatively constant amounts of protein over a wide range of antiserum dilutions. In the ammonium sulfate technique, a constant concentration of globulins is important during the fractionation procedure, because the solubility of these serum proteins in ammonium sulfate varies with their concentration.

Antigen Precipitating Capacity.—A variation of the techniques described by Talmage and Maurer (16) was used to study the precipitating capacity of antisera with I* M_{12}. To 0.5 ml aliquots of serial dilutions of antisera was added 0.5 ml of I* M_{12} antigen. The tubes were incubated at 4°C for 6 days, centrifuged at 2000 R.P.M. for one hour, decanted and the precipitated radioactivity measured. Because of the chemical impurity of the antigen used it was not possible to make quantitative determinations in terms of the amount of antigen nitrogen precipitated by a given amount of antiserum, but it was possible to compare the precipitin reaction and the ammonium sulfate technique in terms of the relative capacities for measuring the anti M protein activity of antisera.

Capillary Precipitin Test.—The method described by Swift *et al.* (17) was used. Readings were taken after incubation for 24 and 48 hours at 4°C and measured according to the criteria suggested by these authors.

Agar Precipitin Test.—A modification of the Ouchterlony technique as described by Halbert (18) was used. The wells were 7 mm in diameter, and the distance between the center well and the peripheral wells was 14 mm. Undiluted antisera and antigen in 0.3 ml quantities were routinely used. Observations were made every other day for 14 days.

RESULTS

Antigenic Nature of I M_{12}.*—To determine whether the iodination procedure or the subsequent attempts to purify the antigen had denatured or significantly altered the properties of the antigen, capillary precipitation and Ouchterlony agar precipitation tests were performed on the acid extract before iodination and on the iodinated test antigen in its most concentrated form. As seen in Table I, capillary precipitin tests gave good reactions with homologous type 12 antisera which had been absorbed twice with a type 17 streptococcus and negative results with similarly absorbed antisera directed against heterologous organisms. The results were essentially the same whether iodinated or uniodinated acid extract was used as the antigen. The differences in the intensity

of the reactions were due to the fact that the iodinated M_{12} antigen used was one-tenth as concentrated as the "purified" uniodinated extract because of dilution occurring in the process of iodination. Agar diffusion plates yielded parallel results in that after absorption of antisera with heterologous streptococci, the iodinated antigen and crude acid extract reacted to form bands of precipitation with homologous antiserum only. Also, the I* M_{12} used in these experiments was sufficiently free of non–type-specific antigen so that when tested by these two methods with unabsorbed heterologous antiserum, no precipitation was observed.

Binding of I M_{12} by Streptococcal Antisera.*—The insolubility of I* M_{12}–globulin complexes in 40 per cent SAS as compared to the solubility of the un-

TABLE I

Capillary Precipitin Tests Performed with Iodinated and Uniodinated Antigen

	Unabsorbed antisera (types)				Absorbed antisera (types)			
	12	14	33	Heterologous multivalent	12	14	33	Heterologous multivalent
Crude acid extract	+++	++	++	++++	++	0	0	0
"Purified" extract prior to iodination*	++++	++	+++	++++	++	0	0	0
I* M_{12}*	+++	0	0	0	++	0	0	0

* In the process of iodination, the antigen was diluted to one-tenth the concentration of the "purified" uniodinated extract.

bound antigen at this salt concentration was used to measure the anti–type 12 antibody activity in a serum. Five-tenths milliliter of I* M_{12} antigen was added to 0.5 ml aliquots of serial dilutions of unabsorbed antiserum. After incubation for 18 hours at 4°C[4], 1 ml of 80 per cent SAS was added to bring the final concentration of ammonium sulfate to 40 per cent. This mixture was allowed to stand at 4°C for one-half hour before centrifugation at 2000 R.P.M. for 30 minutes. After centrifugation, the supernatant fluid was discarded, the precipitate washed in 3 ml of 40 per cent SAS, recentrifuged, decanted, and assayed for radioactivity. Fig. 1 A illustrates the results obtained with unabsorbed hyperimmune homologous and heterologous antisera when per cent radioactivity precipitated is plotted against antiserum dilution. The important points to be noted are: (*a*) the baseline precipitability of the antigen in 1:10 NRS was 11 per cent; (*b*) with 0.05 ml of anti–type 12 antiserum 51 per cent

[4] 4°C is the standard temperature used for incubation, salting out, and centrifugation in these studies. The per cent saturation of ammonium sulfate solutions refers to per cent saturation at 4°C.

of the radioactivity was precipitated in 40 per cent SAS; (*c*) antibody was detected with as little as 0.00008 ml of antiserum; (*d*) with 0.05 ml of unabsorbed hyperimmune heterologous type antiserum 27 per cent of the radioactivity was precipitated in 40 per cent SAS.

In an attempt to decrease heterologous binding, the same experiment as described above was performed on absorbed antiserum. Hyperimmune heterologous antiserum and hyperimmune anti-type 12 antiserum was absorbed with a

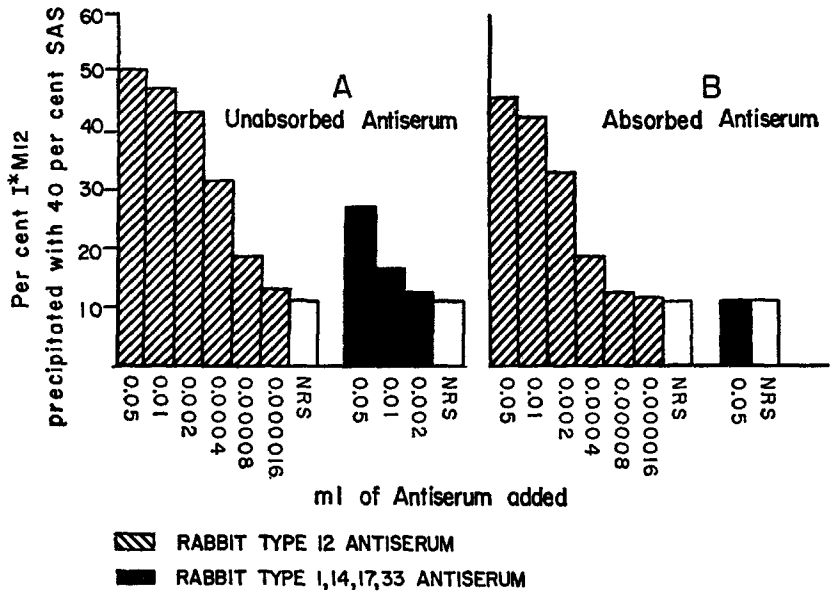
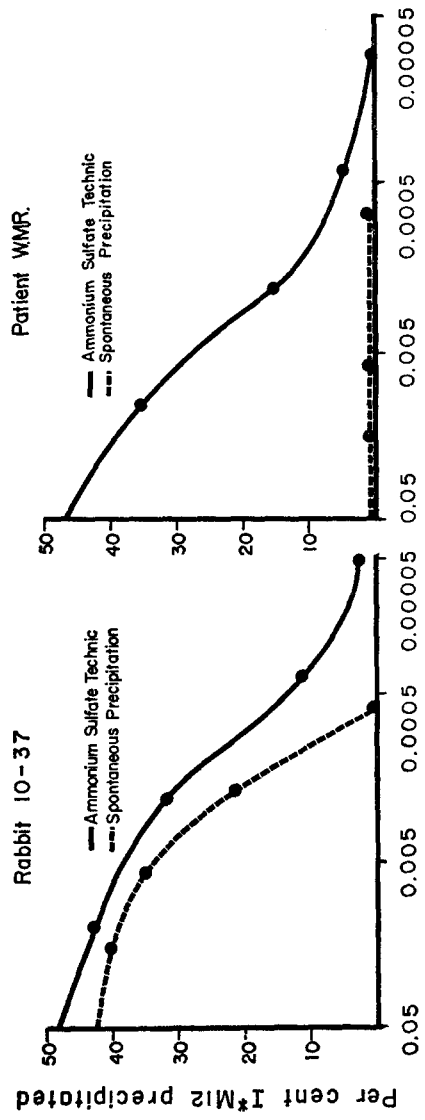


FIG. 1. Binding of I* M_{12} with antiserum directed against type 12 streptococci and heterologous streptococci utilizing absorbed and unabsorbed antiserum.

mixture of type 1 and type 14 heat-killed organisms. One milliliter of packed bacterial sediment was used to absorb 10 ml of a 1:10 dilution of antiserum. Each absorption was carried out for 8 hours at 4°C and centrifuged at 2500 R.P.M. for 30 minutes. One absorption was performed on the heterologous antiserum and three successive absorptions on the homologous anti-type 12 antiserum. Figure 1 B represents the precipitation of I* M_{12} with these sera after the addition of 40 per cent SAS. Whereas one absorption was sufficient to totally eliminate the precipitation caused by heterologous antiserum, three successive absorptions of the homologous antiserum left sufficient antibody so that increased precipitability of I* M_{12} was still observed with a 0.0004 ml of antiserum.

Comparison between Precipitating Capacity and Binding Capacity of Anti-



FIGS. 2 A and 2 B. Comparison between spontaneous precipitation and ammonium sulfate technique in their capacity to detect types pecific anti-M_{12} antibody.

sera.—Hyperimmune absorbed rabbit anti-type 12 antiserum and an absorbed antiserum from a human with rheumatic fever whose serum conferred mouse protection against 2000 times an LD_{50} dose of type 12 streptococcus[5] were used for this experiment. Five-tenths milliliter aliquots of serial dilutions of antisera were added to 0.5 ml of I* M_{12}. One set of aliquots was used for ammonium sulfate precipitation and one set was incubated at 4°C for 6 days and tested for spontaneous precipitation as described previously. The results comparing the two methods of antibody measurement are shown in Fig. 2. While both antisera showed excellent antibody responses as measured by the ammonium sulfate

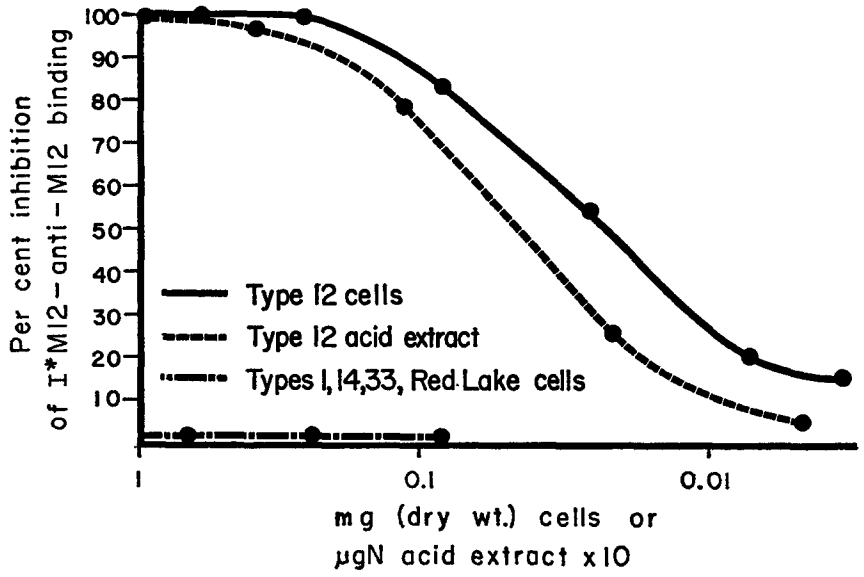


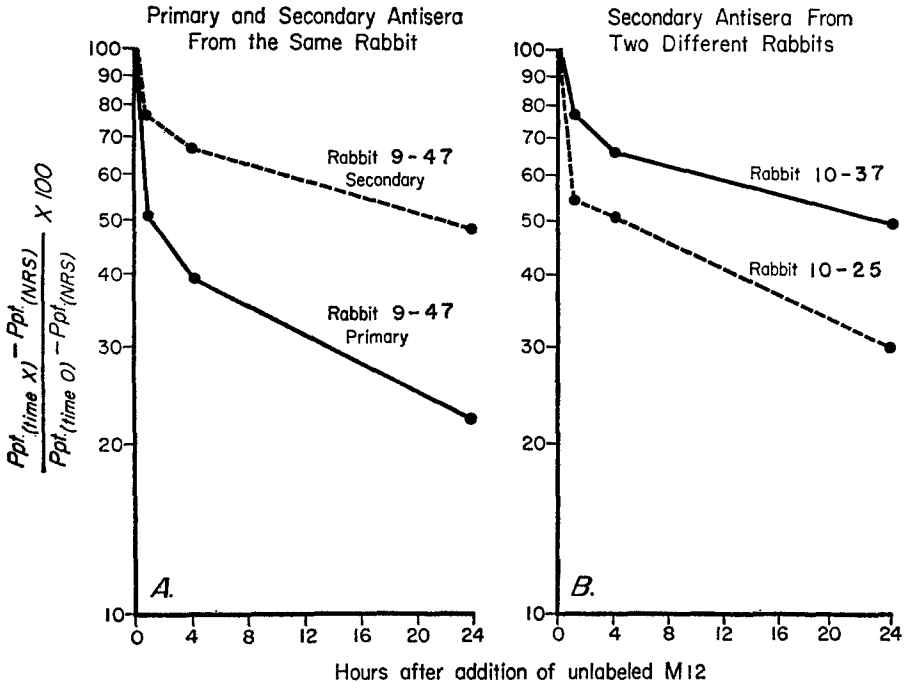
FIG. 3. Type-specific inhibition of I* M_{12}–anti-M_{12} binding by streptococcal cells and acid extracts.

technique, only the rabbit antiserum had any detectable spontaneous precipitin reaction.

Type Specific Inhibition of I M_{12}–anti-M_{12} Binding by Heat-Killed Streptococci and Acid Extracts.*—Unlabeled homologous M protein added to a mixture of I* M_{12} and anti-type 12 antiserum in the zone of antigen excess should compete with and inhibit the complexing of I* M_{12} by anti-M_{12} sites. Under these conditions the extent of the inhibition of the binding between I* M_{12} and anti-M_{12} should be proportional to the quantity of unlabeled M_{12} present; and furthermore, unlabeled heterologous antigens should not inhibit this binding of I* M_{12} to its antibody. To test these propositions, 0.5 ml aliquots of a concen-

[5] Mouse protection tests were kindly performed by Dr. A. I. Braude.

tration of type 12 antiserum, which would bind approximately 35 per cent of the I* M_{12} was added to 0.5 ml aliquots of serial dilutions in 1:20 NRS of uniodinated acid extracts or whole heat-killed streptococci. After 2 hours at room temperature, 0.5 ml of I* M_{12} was added, the mixture allowed to incubate overnight at 4°C, and the procedure described above for precipitation of complexes with 40 per cent SAS carried out. Fig. 3 illustrates that (*a*) the inhibition



FIGS. 4 A and 4 B. Rates of dissociation of I* M_{12}–anti-M_{12} complexes. Graph A represents the rate of dissociation of primary and secondary antiserum from a single animal and graph B repersents the dissociation rates of antisera from two animals immunized and bled at the same time with the same streptococci in adjuvant mixture.

of I* M_{12}–anti-M_{12} binding by M_{12} acid extracts and type 12 organisms was type-specific in that heterologous organisms did not inhibit this binding at all; and (*b*) extremely small quantities of type 12 organisms or type 12 acid extract were capable of totally inhibiting the binding between I* M_{12} and anti-M_{12}.

Dissociation of I M_{12}–anti-M_{12} Complexes.*—When equilibrium is established between unbound antigen and antibody and antigen-antibody complexes, association and dissociation of complexes occur at equal rates. A means of distinguishing between these two opposing processes is necessary before it is possible to study effectively the dissociation of I* M_{12}–anti-M_{12} complexes. This can be

accomplished by the addition of a large excess of unlabeled antigen after equilibrium with isotopically labeled antigen is established, thereby making the relatively small quantities of I* M_{12} present insignificant in terms of future association between antigen and antibody. Any subsequent dissociation could then be studied and would be manifested by a decrease in the amount of I* M_{12}–globulin complexes precipitated upon addition of 40 per cent SAS as compared to the amount precipitated under similar conditions prior to the addition of the unlabeled antigen.

Five-tenths milliliter aliquots of I* M_{12} solution and type 12 antiserum were allowed to equilibrate overnight at 4°C in the region of antigen excess. To these mixtures were added 0.5 ml aliquots of unlabeled M_{12} extract at a concentration approximately 100 times that of the I* M_{12}. This reaction mixture was incubated at 37°C, and at selected time intervals separate aliquots were precipitated with 40 per cent SAS. Fig. 4 illustrates the curves obtained by plotting per cent dissociation

$$\frac{\text{I* in precipitate at time X} - \text{I* in 1:10 NRS precipitate}}{\text{I* in precipitate at time 0} - \text{I* in 1:10 NRS precipitate}} \times 100$$

as a function of time after the addition of unlabeled antigen. As indicated by the steep slope of the first portion of each curve, some of the I* M_{12}–anti-M_{12} complexes dissociated very rapidly. The relative amounts of this quality of antibody varied in the serum obtained at different times during the course of immunization of animal 9-47 (Fig. 4 A) and during the course of immunization of all other animals similarly studied. Thus, 19 days after a single injection of heat-killed organisms, 50 per cent of the antigen-antibody complexes were dissociated in one hour, whereas after secondary stimulation the 50 per cent dissociation time was 21 hours.

Just as the quality of antiserum produced varied within the same animal at different stages of immunization, variations between individual animals of the same species at the same stage of immunization have also been observed. Three monthly injections of heat-killed organisms in adjuvant were administered to each of five animals, and bleedings were taken one week after the last injection. As can be seen from the dissociation rate curves in Fig. 4 B the variations in dissociability of I* M_{12}–anti-M_{12} complexes between the two animals that showed the greatest variations in dissociation times was not as great as was observed between primary and secondary antisera taken from the same animal. Considerable differences were observed, however, in that the 50 per cent dissociation time for the antigen-antibody complexes formed from antiserum obtained from animal 10-28 was 5 hours, whereas the antiserum from animal 10-37 had a 50 per cent dissociation time of 24 hours. The possible biological significance of these differences in the quality of antiserum produced will be discussed below.

DISCUSSION

The precipitation of antigen-antibody complexes in 40 per cent saturated ammonium sulfate was based on the work of Farr who studied a BSA–anti-BSA system (14). In the BSA system, 50 per cent SAS was used to precipitate all of the antibody-bound BSA. However, due to the inherent insolubility of M protein at this salt concentration, it was necessary in the present study to use 40 per cent SAS as the precipitating salt concentration. Not all globulins were precipitated at this concentration, however, and it is probable that under the conditions employed there were antigen-antibody complexes that remained in solution. In the BSA–anti-BSA system approximately 15 per cent of the antigen-antibody complexes formed in the region of antigen excess that were precipitable with 50 per cent SAS remained in solution when 40 per cent SAS was used as the precipitating salt concentration (19). Despite this limitation, the use of ammonium sulfate to precipitate antigen-antibody complexes yielded a system sufficiently sensitive to detect the presence of antibody which was not detectable by the spontaneous precipitin reaction.

The use of isotopically labeled antigen did not appear to affect the serologic characteristics of the antigen as determined by precipitin tests and did make possible the detection of extremely small quantities of antibody. A comparison between the capillary precipitin test and the precipitin reaction utilizing isotopically labeled antigen serves to illustrate this point. Whereas a 600-fold dilution of a hyperimmune antiserum yielded positive results for anti–type 12 antibody when I* M_{12} was used as antigen (Fig. 2 A), a tenfold dilution of the same antiserum yielded negative results in the capillary precipitin test. Although the sensitivity of the I* M_{12} spontaneous precipitin test is slightly less than that exhibited by the ammonium sulfate technique, it would still be a valuable method for the detection of anti-M_{12} antibodies, except for the considerable disadvantage that some sera having relatively large quantities of biologically active antibody, as measured by passive protection and the ammonium sulfate method, had little or no capacity to precipitate antigen spontaneously (Fig. 2 B). This latter observation has been seen in other antigen-antibody systems (14) and has been confirmed in the I* M_{12}–anti-M_{12} system with several other human sera and serves to point up the difficulty that is encountered in the evaluation of results obtained from any test that deals with secondary manifestations of antigen-antibody binding.

In all measurements of anti-M protein antibody it is imperative to eliminate to as great an extent as possible non–type-specific antigen-antibody interaction that might be mistaken for M protein–anti-M protein activity. This can be accomplished by either of two methods: (1) purification of the antigen so that M protein is the only antigen present; (2) absorption of non–type-specific antibody from the antiserum. Both methods were used for the present study. The antigen was purified by incubation with heterologous antiserum and pre-

cipitation with ammonium sulfate to dispose of most of the antigen molecules capable of binding with non-type-specific antibody. The antisera were also purified by absorption with heterologous organisms. The end result of these procedures was a test system which had no reactivity with absorbed heterologous antisera and in which up to 50 per cent of the radioactivity was specifically precipitated by anti-type 12 antiserum.

The binding exhibited by unabsorbed heterologous antiserum and I* M_{12} may have been due to two mechanisms: (1) the presence of contaminating non-type-specific antigen-antibody systems; (2) cross-reactivity between type 12 M protein and a heterologous anti-M antibody. As in other studies that have involved this antigen-antibody system (5, 8, 10, 20), it was not possible to determine with any certainty which of these mechanisms was operative. It appears likely that this particular problem will be solved only when M antigen is obtained in a chemically purified state. Until such time, however, it appears necessary to resort to heavy absorption of test antisera with heterologous organisms despite the possibility that biologically important antibodies produced against the M protein of one type may be removed by absorption with heterologous organisms due to cross-reactivity between the homologous M protein and the heterologous M protein used for absorption.

Recent studies (21) utilizing the bactericidal test and long chain reaction to detect M protein have shown that these tests can detect approximately 0.08 and 0.3 μg of soluble M protein respectively. In the I* M_{12}–anti-M_{12} system, 0.005 μg of protein nitrogen or 0.03 μg of protein was sufficient to inhibit the reaction by 50 per cent (Fig. 3). This capacity to detect these small quantities of M protein may provide a useful tool in investigations concerned with studying the role of streptococcal antigens in the pathogenesis of tissue lesions seen in the non-suppurative complications of streptococcal infection. The I* M_{12}–anti-M_{12} system can also be used to quantitate the M protein content on the surface of different strains of streptococci. Preliminary studies from this laboratory indicate that equally virulent strains of type 12 streptococci may differ by several fold in the surface content of M protein. Also, an individual strain which has become avirulent by subculture onto artificial media may, on occasion, contain more surface M protein than the virulent parent strain. These results add some evidence to the hypothesis that although the ability of an organism to produce M protein is probably a prerequisite for human or mouse virulence, other undetermined factors must also be operative before an organism can obtain maximal virulence (3).

The capacity of primary binding tests to yield data which characterize quality as well as quantity of antibody adds another parameter with which to study immune phenomena of M protein and its antibodies. The importance of this aspect of the immune mechanism in terms of biological function has been demonstrated by Jerne in the neutralization of diphtheria toxin by anti-

toxin (22) and by Talmage (23) in the red cell–hemolysin complement systems. The possible role that the quality of the antibody to M protein has on such biological phenomena as the type-specific protection of antisera against streptococcal infection and the production of acute glomerulonephritis in humans is under investigation.

SUMMARY

A method for detecting type-specific antibody to type 12 β-hemolytic streptococci is described. The method is based on the utilization of a partially purified I^{131} labeled streptococcal acid extract and the solubility of this extract in 40 per cent saturated ammonium sulfate as compared to the insolubility of antibody and antigen-antibody complexes at this salt concentration.

The salient features of this technique are:

(*a*) When absorbed sera were used, no non–type-specific reaction with antigen occurred.

(*b*) The sensitivity of the system allowed detection of type-specific antibody in hyperimmune antisera that had been diluted 1200-fold.

(*c*) The method does not rely on secondary manifestations of antigen-antibody interaction for the detection of antibody.

(*d*) As little as 0.03 μg of M_{12} protein was detected when soluble unlabeled M protein was used to inhibit the reaction between I^{131} M_{12} protein and anti-M_{12} antibody.

(*e*) The kinetics of the reaction between M protein and anti-M antibody can be studied, thereby making information available as to the quality as well as the quantity of antibody produced in this antigen-antibody system.

The author gratefully acknowledges the encouragement and advice of Dr. Richard S. Farr in whose laboratory this work was done. The able technical assistance of Miss Winnie Dickens and Mr. Richard Mays is also gratefully acknowledged.

BIBLIOGRAPHY

1. Todd, E. W., and Lancefield, R. C., Variants of hemolytic streptococci; their relation to type specific substance, virulence and toxin, *J. Exp. Med.*, 1928, **48,** 751.
2. Lancefield, R. C., and Todd, E. W., Antigenic differences between matt hemolytic streptococci and their glossy variants, *J. Exp. Med.*, 1928, **48,** 769.
3. Rothbard, S., Protective effect of hyaluronidase and type-specific anti-M serum on experimental group A streptococcus infections in mice, *J. Exp. Med.*, 1948, **88,** 325.
4. Rammelkamp, C. H., and Weaver, R. S., Acute glomerulo-nephritis. The significance of the variations in the incidence of the disease, *J. Clin. Invest.*, 1953, **32,** 345.
5. Lancefield, R. C., The antigenic complex of *Streptococcus haemolyticus*. I. Demonstration of a type specific substance in extracts of *Streptococcus haemolyticus*, *J. Exp. Med.*, 1928, **47,** 91.

6. Griffith, F., Serologic classification of *Streptococcus pyogenes*, *J. Hygiene*, 1934, **34,** 542.
7. Bone, M., Braude, A. I., Kleinman, H., Complement fixing antibody response to M-protein of nephritogenic streptococci in glomerulonephritis, *J. Lab. and Clin. Med.*, 1957, **50,** 705.
8. Hirst, G. K., and Lancefield, R. C., Antigenic properties of the type-specific substance derived from group A hemolytic streptococci. *J. Exp. Med.*, 1939, **69,** 425.
9. Rothbard, S., Bacteriostatic effect of human sera on group A streptococci. II. Comparative bacteriostatic effect of normal whole blood from different animal species in the presence of human convalescent sera, *J. Exp. Med.*, 1945, **82,** 107.
10. Lancefield, R. C., and Perlman, G. E., Preparation and properties of type-specific M antigen isolated from a group A, type 1 hemolytic streptococcus. *J. Exp. Med.*, 1952, **96,** 71.
11. Lanni, F., Dillon, M. L., Beard, J. W., Determination of small quantities of nitrogen in serological precipitates and other biological materials, *Proc. Soc. Exp. Biol. and Med.*, 1950, **74,** 4.
12. Talmage, D. W., Baker, H. R., and Akeson, W., The separation and analysis of labeled antibodies, *J. Infect. Dis.*, 1954, **94,** 199.
13. Lancefield, R. C., A micro precipitin technic for classifying hemolytic streptococci, and improved method for producing antisera. *Proc. Soc. Exp. Biol. and Med.*, 1938, **38,** 474.
14. Farr, R. S., A quantitative immunochemical measure of the primary interaction between I*BSA and antibody. *J. Infec. Dis.*, 1958, **103,** 239.
15. Adams, M. H., Surface inactivation of bacterial viruses and of proteins, *J. Gen. Physiol.*, 1947, **31,** 417.
16. Talmage, D. W., and Maurer, P. H., I^{131} labeled antigen precipitation as a measure of quantity and quality of antibody. *J. Infect. Dis.*, 1953, **92,** 288.
17. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes, *J. Exp. Med.*, 1943, **78,** 127.
18. Halbert, S. P., Swick, L., and Sonn, C., The use of precipitin analysis in agar for the study of human streptococcal infections. II. Ouchterlony and Oakley technics, *J. Exp. Med.*, 1955, **101,** 557.
19. Farr, R. S., unpublished data.
20. Pierce, W. A., Jr., Agar diffusion studies of group A streptococcal antigens, *J. Bact.*, 1959, **77,** 726.
21. Ekstedt, R. D., and Stollerman, G. H., Factors effecting the chain length of group A streptococci. II. Quantitative M-anti-M relationships in the long chain test, *J. Exp. Med.*, 1960, **112,** 687.
22. Jerne, N. K., A study of avidity. *Acta Path. et Microbiol. Scand.*, 1950, suppl. 87, 1.
23. Talmage, D. W., The primary equilibrium between antigen and antibody, *Ann. New York Acad. Sc.*, 1957, **70,** 82.