NEW APPROACHES FOR THE LABORATORY RECOGNITION OF M TYPES OF GROUP A STREPTOCOCCI*

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The antigenic classification of Group A hemolytic streptococci is based on the occurrence of multiple M and T antigens (1, 2). Epidemiological studies on the transmission of streptococci in the population are largely dependent on the recognition of these antigens in the isolated strains. As a general rule, a streptococcal strain reacts in the precipitin test with a single absorbed anti-M serum, but it is commonly agglutinated by multiple anti-T sera. Immunity to infection with Group A streptococci is determined by the immune response to the individual M antigens (2). Therefore, the specificity of the M reaction has special significance for epidemiological studies.

The successful classification of streptococci requires a complete series of antisera which are sufficiently potent and specific to give unequivocal type-specific precipitin reactions with all the serotypes. There are now more than 50 M serotypes. The necessity of preparing a large number of absorbed type-specific antisera suitable for this purpose has been circumvented by using unabsorbed anti-M sera and crude HCl extracts in the Ouchterlony double-diffusion test, as first suggested by Michael and Massell (3). This technique, which could well have been employed earlier, is now examined in detail here. It represents a departure from the established procedure of using specifically absorbed antisera.

In addition, a method was developed to use an immunoabsorbent column containing the chemically defined immunodominant determinant of the Group A carbohydrate coupled to an insoluble Sepharose matrix in order to absorb the Group A antibodies, the major cross-reacting antibodies, from those antisera which could not be used unabsorbed (4). This absorption method is ap-

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plicable to the preparation of antisera to be used for M typing either by the standard capillary precipitin test or by the Ouchterlony double-diffusion method.

Materials and Methods

Standard Strains of Streptococci.—Representative streptococcal strains of all M types which are available at The Rockefeller University laboratory were employed in these studies to prepare standard HCl extracts and vaccines. These strains are as follows

Type 1, T1/195/2; Type 2, T2/44/RB4/119; Type 3, B930/60/2; 3R, D58X; Type 4, T4/95/RB5; Type 5, T5B/126/3; Type 6, S43/192/1; Type 8, C265/86/1; Type 9, T9/120/1; Type 11, T11/137/1; Type 12, T12/126/4; Type 13, T13/150/2; Type 14, T14/46/7; Type 15, T15/32/6; Type 17, T17E/165/1; Type 18, T17C/55/2; Type 19, J17D/70/6; Type 22, T22/76/3; Type 23, T23/70/RB5; Type 24, C98/105/2; Type 25, B346/94/3; Type 26, J17F/123/1; Type 27, Type 27; Type 28, T28/150A/4; 28R, B337; Type 29, D23; Type 30, D24/126/2; Type 31, T137/69/1; Type 32, C121/39/8; Type 33, C107/24/8; Type 34, C142/82/5; Type 36, C119/83/1; Type 37, C242; Type 38, C94/80/1; Type 39, C95/60B/3; Type 40, C143/25/7; Type 41, C101/103/2; Type 42, C113/55/3; Type 43, C126/59/6; Type 46, C105/41/6; Type 47, C774/RB4/6/4; Type 48, B403/48/4; Type 49, B737/71/2; Type 50, B514/33/5; Type 51, A309/31/5; Type 52, A871/14/3; Type 53, A952/94/1; Type 54, A953/87/1; Type 55, A928; Type 56, A963; Type 57, A995; Type 58, D315/87/1; Type 59, D307; Type 60, D335; and Type 61, D336/56/1.

Field Strains.—Freshly isolated strains from clinical material and from epidemiological studies were obtained from The Rockefeller University collection; from Drs. L. W. Wannamaker and S. S. Chapman, University of Minnesota, Minneapolis; from Dr. J. Sramek, Institute of Epidemiology and Microbiology, Prague; and from Dr. Richard Roberts, Cornell University, New York Hospital Medical Center, New York.

M-Type Antisera.—Hyperimmune anti-M antisera were prepared in rabbits immunized with the standard streptococcal strains listed above (see Appendix). These antisera were employed either unabsorbed or specifically absorbed. Before use, it was determined that the sera did not contain antibody to polyglycerol phosphate (5, 6).

Capillary Precipitin Tests.—The capillary test procedure described by Swift et al. was used (7).

Streptococcal Group-Specific Carbohydrate.—The preparation of Group A carbohydrate was previously described (8).

Quantitative Precipitin Test for Antibodies to Group-Specific Carbohydrate.—The previous method for quantitative precipitin analysis has been modified so that the total protein in the immune precipitate dissolved in sodium hydroxide is measured in a Technicon autoanalyser (Technicon Co., Inc., Tarrytown, N. Y.) which has been adapted to perform the Lowry protein determination (9).

M Extracts.—The usual crude extracts prepared by heating the streptococci at 100°C in HCl, pH 2, were used whenever possible in serological tests for M typing (10).

For a partially purified M preparation, the M protein could be conveniently separated from the group polysaccharide by reprecipitating it two or three times with 3 vol of cold ethanol, or until a solution of the M protein no longer precipitated group-specific antisera but still gave maximal precipitin reactions with absorbed type-specific antisera (10).

A somewhat more purified M protein than the one described above was prepared by the method of Lancefield and Perlmann (11). The Type 12 streptococci were collected from 20 liters of broth, washed, and extracted with HCl adjusted to pH 2 at 100°C. After purification the final yield of M protein was 50 mg. Chemical analysis indicated that the protein content

was 90% of the final frozen and dried product. This protein preparation gave no or, at most, minimal cross-reactions with heterologous antisera.

Quantitative Precipitin Test for Antibodies to M Protein.—A quantitative precipitin test was developed for measuring the anti-M antibodies in the antisera before and after absorption.

Fig. 1 shows the results of a quantitative precipitin test between the more purified Type 12 M protein made up by weight for a standard solution and unabsorbed antisera of Types 6,

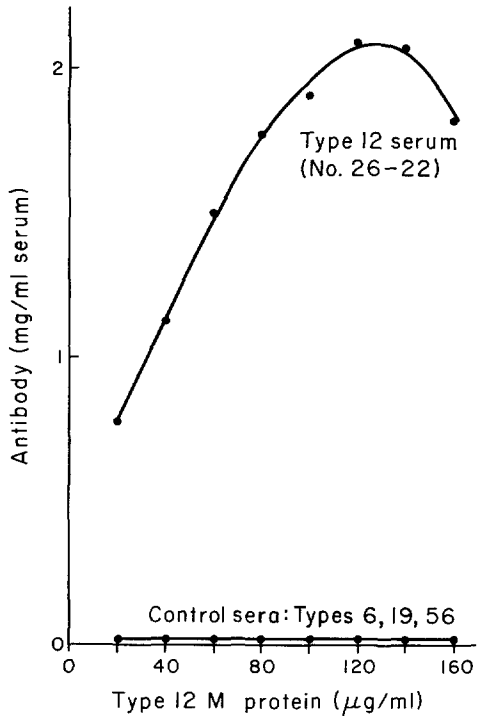

FIG. 1. Quantitative precipitin tests with purified Type 12 M protein and homologous unabsorbed antisera. Types 6, 19, and 56 antisera were used as controls.

12, 19, and 56. The protein in the immune precipitate was determined by the same method employed for the quantitative precipitin test for the antibodies to Group A carbohydrates (9).

Immunoabsorbents.—Two kinds of immunoabsorbents were prepared, either of which could be used to remove the cross-reactive antibody to the Group A group-specific substance. Sepharose 2b was employed as the insoluble matrix. The first immunoabsorbent was prepared by coupling para-aminophenyl-β-*N*-acetylglucosamine to cyanogen bromide–activated Sepharose (12). This batch was kindly supplied by Dr. David Parker, University of California, Berkeley, Calif. (4).

A brief description of the preparation of a batch of immunoabsorbent made in the same way follows. 100 ml of packed Sepharose 2b was washed with distilled water. The washed Sepharose was added to 100 ml of distilled water in which 5 g cyanogen bromide had been dissolved. The pH was raised immediately to pH 11 with 4 N NaOH and this pH was maintained

while stirring at 20°C. The reaction was complete in about 10 min. A stable pH without added NaOH is an indication of completion of the reaction. Very quickly the solution was rinsed into a sintered glass funnel and washed with 20 vol of cold (4°C) buffer, 0.1 M Na_2CO_3, at pH 9. The rinsed, activated Sepharose was transferred to a beaker in which 1.25 mg (4 μmole) of para-aminophenyl-β-*N*-acetylglucosaminide (Cyclo Chemical Corp., Los Angeles, Calif.) had been dissolved in a small amount of buffer. The total volume was brought to 200 ml and the mixture was stirred at room temperature for 24 hr. The final product was washed with 20 vol of distilled water and then with 20 vol of the buffer to be employed on the immunoabsorbent column. The capacity of this batch of immunoabsorbent was 20 mg of Group A antibody/ml of packed Sepharose.

In the second procedure the immunoabsorbent was prepared from Group A carbohydrate instead of *N*-acetylglucosamine, as above. 100 mg of Group A carbohydrate which had been partially deacetylated to permit combination with cyanogen bromide–activated Sepharose was treated by the same method employed previously to prepare a Group C immunoabsorbent (13, 14).

M Typing Using Double-Diffusion Precipitin Reaction in Agar.—These tests were performed by the usual methods (15). Six glass microscope slides were held in place with a plastic frame manufactured for this purpose by Gelman Instrument Company, Ann Arbor, Mich. 12 ml of melted Noble agar in 0.01 M phosphate-buffered 0.85% NaCl, pH 7, containing 0.01% sodium azide, was added to the frame and, after solidification occurred, two circular sets of wells (eight peripheral wells and one central well) were cut in each slide by the device also manufactured by Gelman Instrument Company. The distance from the center of the central well to the center of a peripheral well was 7.5 mm. The diameter of the wells was 3 mm.

In the performance of the double-diffusion test for M typing of streptococcal extracts, the unabsorbed antisera were placed in the central well, and eight crude HCl extracts in the peripheral wells, including the homologous type, with each serum. Thus, 53 circular sets of wells were needed to test the extracts against all 53 antisera. The tests were kept at room temperature overnight and read.

If only a single extract was to be identified, it was still necessary to prepare all 53 sets of wells with a different antiserum in each central well. Economy of time could not be achieved by placing a single extract in several central wells and antisera in the eight peripheral wells of the several sets. Such a procedure leads to numerous cross-reactions, presumably because the larger serum volume on the plate increases the concentration of cross-reacting antibodies to the point where they give visible reactions with non–M protein antigens in the extract.

EXPERIMENTAL

In the present experiments streptococcal anti-M unabsorbed sera of 53 different serotypes were examined by double-diffusion tests in parallel with capillary precipitin tests using absorbed antisera. As specificity controls in all of these double-diffusion tests, each serum was tested against crude HCl extracts of all 53 streptococcal types.

In this initial attempt to use the double-diffusion test with unabsorbed anti-M serum and crude HCl extracts, the first selective process used to obtain suitable sera was a presumptive precipitin test for the presence of M antibodies. The most potent unabsorbed sera were employed from rabbits immunized for the preparation of stock anti-M precipitin typing sera. It was found that the type antisera which gave good anti-M precipitins in the capillary test were also successful in Ouchterlony double-diffusion M typing. Only about one-third of those used in this experiment contained appreciable concentrations of group-specific antibodies to

the cell wall polysaccharide, although antisera free of this antibody were found later by screening other batches of antisera which were available. In many cases this group antibody did not interfere with the recognition of the type-specific M reaction, because of the different diffusion rates in the agar gel of the fast-moving group polysaccharide and the more slow-moving M protein, for example, Types 1, 6, and 17 (Fig. 2).

Although all of the homologous reactions are shown in Fig. 2, only a limited number of the representative heterologous tests performed in the double diffusion experiments could be depicted in this figure. For 41 of the 53 sera, an unmistakable homologous reaction occurred which could be readily distinguished from all minor cross-reactions with heterologous extracts. In all of these cases the reaction with the group-specific carbohydrate, when present, was near the central antiserum well and did not interfere with the recognition of the type-specific reaction. Several antisera employed here, notably Types 9, 22, 27, 34, 38, 42, and 59, were either too weak to give unequivocal reactions in these types or else cross-reactions blurred specific identification, but this was also the case in the standard capillary precipitin test. All of the other antisera produced either strong or adequate precipitin bands.

The type-specific identification of these streptococci with unabsorbed antisera by the Ouchterlony method was in agreement with the results obtained with these same extracts in the capillary precipitin test using absorbed antisera.

In the use of this Ouchterlony method for the identification of the M type of streptococci, confusion may arise if the reactions produced by the R antigen are not taken into consideration (16). For example, the Type 28 band in Fig. 2, formed with the particular unabsorbed serum used here, identified the non-type-specific 28R antigen (previously described in detail [16, 17, 22], and also present in M-Type 28 streptococci) because this serum contains little or no M-Type 28 antibody. The absorbed Type 28 anti-M serum has always been weak, and so far often unsatisfactory for identifying the 28M antigen by either method, whereas most sera contain appreciable amounts of antibodies to the non-type-specific 28R antigen.

In the Type 50 of Fig. 2, most of the precipitin band is probably due to the previously undescribed R antigen of Type 50 which, as well as Type 50 M antigen, is also specific for Type 50. It is a prominent part of the cell surface of these strains. Another known R antigen, in this case having Type 3 specificity, occurs in about one-half of the Type 3 strains (18) and has not been found elsewhere. Some newly described R antigens with Type 42 specificity have also been studied by Wiley and Bruno (19).

Cross-reactions other than those due to the R antigens, which might be mistaken for the M-anti-M reaction, were rare. This was probably because of the reduction by dilution of the cross-reactive concentration of antibodies below the threshold for precipitation as they diffuse from the central serum well through the agar gel.

Cross-reactions, when they did occur, usually reflected the ractivity of a particular serum. When several unabsorbed antisera for a given type were tested, one could usually be found which gave no troublesome cross-reactions. Such data are shown in Table I. Serum 53a of M Type 53, for example, gave

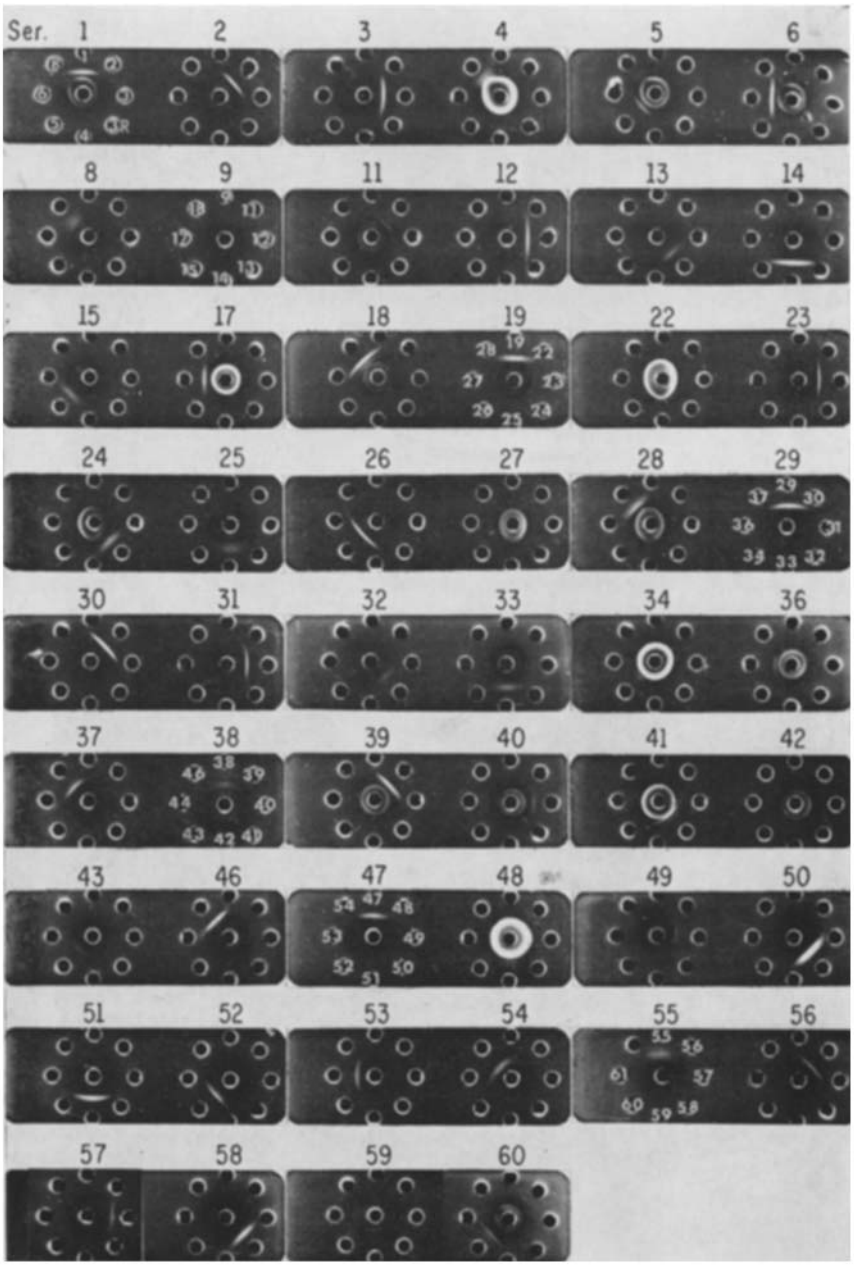

FIG. 2. Ouchterlony double-diffusion tests with crude HCl extracts and unabsorbed M-type antisera. Extracts of Types 1, 2, 3, 3R, 4, 5, 6, and 8 streptococci were placed in the outer wells of the matrices which contained in the center wells sera to M Types 1, 2, 3, 4, 5, 6, and 8. A similar protocol was employed for the other serotypes. Only representative examples of the matrices which illustrate the patterns of the reactions and were employed to test all of the 53 extracts with all the 53 antisera are shown here. Antisera which had high concentrations of group-specific antibody gave a heavy inner ring of precipitate; for example, antisera 4, 22, 34, 41, and 48. The specific precipitin line due to the M protein can be differentiated from the carbohydrate reaction, for example, antisera 5, 6, and 17.

cross-reactions with extracts of M Types 8, 43, 52, and 56. These cross-reactions were of such intensity that the corresponding strains could be erroneously identified as M Type 53, if this antiserum were employed. On the other hand, M Type antisera numbers 53b and 53c did not cross-react with any heterolo-

TABLE I

Typical Examples of Cross-Reactivity of Sera from Different Rabbits. Selection of Best Unabsorbed Antisera for M Typing by Double-Diffusion Technique. Results of Double Diffusion in Agar Gel

Unabsorbed antisera		Intensity of reactions			Comment
		Crude HCl extracts			
M type	Different rabbits and batches Lot No.	M band with homol. type Type-spec. reaction	M-like band looked for with 52 heterol. types Cross-reactions found*	Group A-CHO Group band Group reactions‡	Best selection of sera usable for M typing Without absorption
3	3a	+++	Types 25±, 31+, 59±	±	Not usable
	3b	+++	0	+++	Usable
	3c	+	0	+++	Not usable
	3d	+++	0	0	Preferred serum
	3e	++	0	+	Usable
31	31a	+++	Types 3±, 8±	+++	Not usable
	31b	+++	0	0	Preferred serum
	31c	+++	Type 3++	+++	Not usable
	31d	+++	Types 3++, 25±	0	Not usable
	31e	+++	Types 3++, 25+	0	Not usable
53	53a	+++	Types 8±, 43±, 52++, 56++	0	Not usable
	53b	+++	0	0	Preferred serum
	53c	+++	0	0	Preferred serum
	53d	+++	Type 52±	0	Not usable
	53e	+++	0	0	Preferred serum

* Sera with strong cross-reaction cannot be used without absorption with heterologous streptococcal cells.

‡ Sera showing homologous Group A-CHO reactions can usually be employed without absorption or after-immunocolumn absorption (*N*-acetylglucosamine). See figures.

gous extract in a fashion that would lead to confusion in the recognition of the type-specific precipitin band. Only one substitution (Type 3) was made in the experiment illustrated in Fig. 2, and the panel of sera was thereafter kept unchanged throughout the following study on the identification of field strains.

Extension of the Study to Freshly Isolated Strains.—117 strains were employed in an experiment to determine whether double diffusion in agar gel with unab-

sorbed streptococcal anti-M sera could be used successfully to identify the M antigens of streptococci freshly isolated from hospital patients and in epidemiological studies. Crude HCl extracts were prepared from each strain and capillary precipitin tests were set up against all 53 standard absorbed anti-M sera prepared in our laboratory. Not all of these antisera gave satisfactory precipitin reactions with their homologous strains, but all were included since they were the best available. The double-diffusion tests in agar gel were also set up in parallel to type the same crude HCl extracts prepared from these

TABLE II

Experiment with Field Strains. Comparison of Streptococcus M Typing Using Absorbed Antisera in Capillary Tests and Unabsorbed Antisera in Agar Double Diffusion

Analysis of results	No. of strains		%
Agreement of results			
Complete		104	88.9
Partial		5	4.3
Disagreement of results			
Different types		1	0.8
Typable by capillary tube only		3	2.6
Typable by double diffusion only		4	3.4
Total		117	100.0
Analysis of strains showing complete agreement by both techniques			
M typed*		47	45.0
M untypable			
1) 28R strains probably‡ indicating M Types 2, 28, or 48	9	57	55.0
2) No indication of type	48		
Total		104	100.0

* One 28R strain with Type 2 M antigen was identified. Type 2 strains with 28R antigen occur frequently, although most Type 2 strains do not have this antigen.

‡ For the three types which may contain 28R antigen in some strains, satisfactory high titer anti-M sera are not available at present.

freshly isolated strains with the same unabsorbed antisera used to identify the control standard type strains tested by the double-diffusion technique (Fig. 2).

Tables II and III summarize the results of typing these 117 strains. The precipitin typing with absorbed anti-M serum and the agar double diffusion with unabsorbed antisera showed very little difference in results, with only one instance of different types indicated by the two methods. The M type was identified for 60–65% of the strains from New York Hospital, The Rockefeller University, and the University of Minnesota. The scattering of types usually observed in sporadic infections in civilian populations also seemed to hold in

these small samples from various locales. The small number of identifiable strains among the Prague collection may indicate the occurrence of serotypes in that population for which M type antisera are not available.

As a result of this study, it is thought possible to institute a useful change in the system of M typing, as an auxiliary procedure to the capillary precipitin method, by first testing with the double-diffusion technique described above. Confirmation by capillary precipitin testing with absorbed anti-M serum would

TABLE III

Experiment with Field Strains (M Types Found)

Source of strains Number examined Results	The New York Hospital		The Rockefeller University		Prague		University of Minnesota	
	18		31		28		27	
	M type	No. of strains	M type	No. of strains	M type	No. of strains	M type	No. of strains
M types identified	1	1	1	1	5	1	1	1
	3	2	5	2	18	1	2*	1
	5	1	6	2			5	3
	12	1	9	1			6	2
	41	3	12	7			12	3
	52	1	18	2			29	1
	53	1	19	1			41	1
	56	1	49	1			56	2
	58	1					57	2
M-untypable strains								
1) 28R‡				4		2		3
2) No indication of type		6		10		24		8

* Same as footnote in Table II.

‡ Probably indicating M Types 2, 28, or 48.

check the adequacy of this method as necessary, and permit the subsequent use of the Ouchterlony method alone in large-scale epidemiological studies.

Use of M Antisera Devoid of Antibodies to Group A Carbohydrate. In the initial survey described above, unabsorbed antisera were employed, some of which gave only the M precipitin band, while others gave both the M precipitin and the group carbohydrate precipitin bands. While it is possible to identify the M-type strains with antisera which contain both group and type antibodies, it is obvious that the method would be improved if all of the M type antisera were devoid of the antibody to the carbohydrate. For this reason all available anti-M antisera remaining in our collection were screened to identify those which gave a strong M precipitin band, and either no Group A carbohydrate band or one that was very weak.

All batches of antisera of a given type were screened by the double-diffusion test performed on microscope slides which contained three rows of wells, five wells per row. The distance between the central row of wells and the two outside rows of wells is the same as the distance between the central wells and the peripheral wells in the double-diffusion test (Fig. 2). The antisera were placed in the center row of wells and the homologous M antigen free of group polysaccharide in each well in the bottom row. The purified group polysaccharide was placed in the wells in the top row. The sera could thus be screened for anti-M antibody in the bottom row and in the top row for Group A antibody. Results with three representative M-Type 12 antisera and a control Group A serum are shown in Fig. 3. Unabsorbed antiserum (a) gave both M protein and carbohydrate precipitin bands. Antisera (b) and (c) gave only the M protein band. The control Group A serum showed no M band. This proved to be a rapid and accurate method of ascertaining whether one or both of these antibodies was present in a total of 465 sera representing 53 serotypes. 30 samples of antisera could be screened for these two antibodies in one-half hour or less.

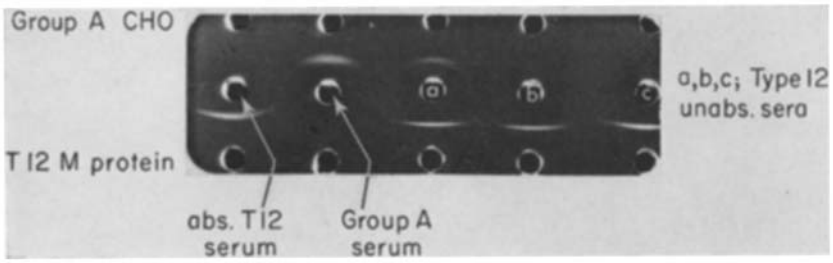

FIG. 3. The double-diffusion procedure which was employed to check unabsorbed M-type antisera for the presence of antibodies to Group A carbohydrate and to M protein. For example, shown here are the reactions between three unabsorbed Type 12 antisera, a, b, and c, and the Group A carbohydrate and Type 12 M protein. Unabsorbed serum (a) reacts with both the carbohydrate antigen and the Type 12 M protein, and, therefore, is not as useful in the Ouchterlony double-diffusion test. Antisera (b) and (c) which did not react with Group A antigen reacted with the Type 12 M protein.

For most serological types, one to four of the available samples of each type of antiserum were found to have no group-specific antibodies demonstrable by this method and to show strong M type specific precipitin bands in the agar double-diffusion slides. This amounted to 20% of all samples tested. Another 32% of the samples were suitable for use after absorption by the immunoabsorbent column procedures (see below) to remove the Group A antibodies. Certain samples could be used without absorption because the concentrations of the Group A antibodies were low. Thus, by selecting from among all of the available sera, sera for 37 of the 53 known M types could be used without any absorption, and representative sera of 12 to 14 other M types could be satisfactorily treated on the immunoabsorbent column. A panel of antisera is now available in which all antisera give only the M-type precipitin line.

It has always been very difficult to prepare potent anti-M sera for several of the types for use in the capillary precipitin reaction, and the same sera were also ineffective in double diffusion.

Serum Absorption with an Immunoabsorbent Column.—The immunoabsorbent column procedure described by Parker and Briles (4) for the isolation of group-specific antibodies from Group A sera has been used in the present study to eliminate these antibodies from anti-M antisera.

The principle involved is the selective absorption of Group A antibodies with an immunoabsorbent, prepared by coupling para-aminophenyl-β-*N*-acetyl-

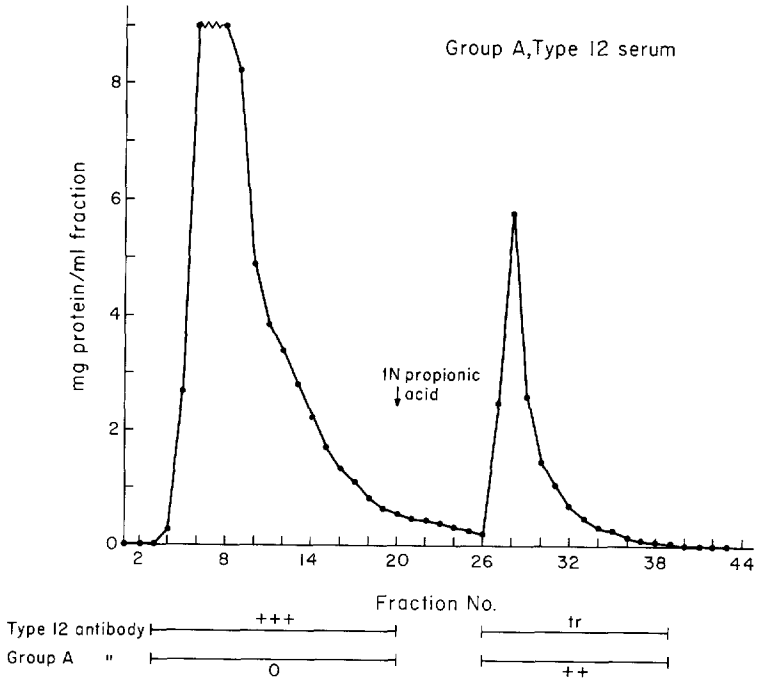

FIG. 4. Absorption of Group A antibodies from an M–Type 12 serum by a specific immunoabsorbent column. The serum components which pass through the column contain Type 12 antibodies and the column holds back the Group A antibodies. The Group A antibodies are then eluted with 1 N propionic acid.

glucosamine to Sepharose 2b activated with cyanogen bromide, which is then used to prepare an immunoabsorbent column (see Materials and Methods). The specific absorption is dependent upon the fact discovered by McCarty (20) that β-*N*-acetylglucosaminide is the terminal immunodominant determinant of the Group A polysaccharide. When the antiserum is passed through the immunoabsorbent column, the Group A antibodies remain attached to the column and the type-specific M antibodies appear in the initial eluate of PO_4-buffered saline.

In a typical experiment illustrated in Fig. 4, 10 ml of unabsorbed Type 12 antiserum was loaded on an immunoabsorbent column (column bed 1.5 × 28 cm). The column bed had been

equilibrated with phosphate-buffered saline (0.02 M PO_4-buffered saline, pH 7, containing 0.01% sodium azide), and the serum proteins not absorbed to the column were washed through with this buffer. The wash was collected in 3-ml fractions. Each fraction was tested for M Type 12 and Group A antibodies by the capillary precipitin test and for protein as described in Materials and Methods. Most of the protein was eluted from the column between fractions 3 and 20. Fraction 20 contained only traces of anti-Type 12 antibody and no Group A antibody. Fractions 2 through 20 were pooled and concentrated by vacuum dialysis to 10 ml, the initial serum volume. To insure sterility, the final product was filtered through a Millipore filter (Millipore Corp., Bedford, Mass.) into a sterile bottle. In a capillary precipitin test, this absorbed serum gave a 3+ precipitin reaction with Type 12 M protein, but no reaction with Group A carbohydrate. The absorbed serum, as determined by quantitative precipitin tests,

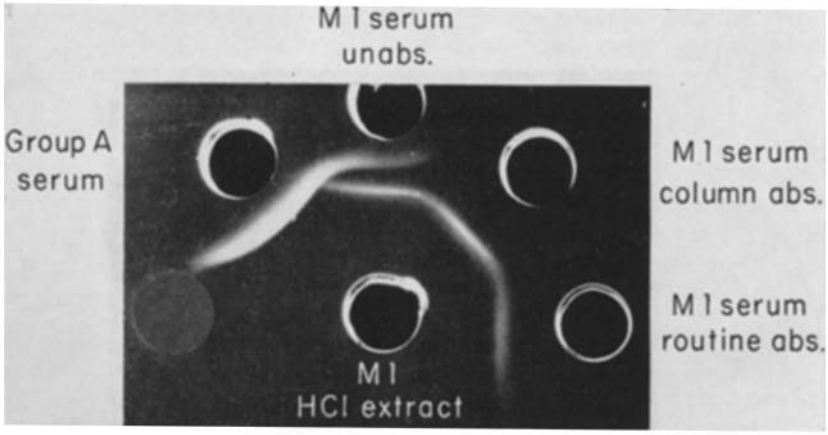

FIG. 5. An Ouchterlony double-diffusion test which shows the Group A carbohydrate and the M protein precipitin lines formed between an unabsorbed M–Type 1 antiserum and an HCl extract of Type 1 streptococci. The Group A precipitin line is not seen either with sera absorbed with the column or with heterologous streptococcal suspensions.

contained 2/3 of the antibody to the M protein which had been found in the unabsorbed serum.[1] It is known, however, that absorption with heterologous streptococci also diminished the content of anti-M antibodies in serum by nonspecific absorption of serum proteins.

After the Group A antibody and other protein was eluted from the column with 1 N propionic acid, it was reequilibrated with phosphate-buffered saline and was ready for repeated use. Because the buffer contained sodium azide, the column could be stored indefinitely at 4°C. The same column has now been used repeatedly for over 1 yr. Deterioration of the immunoabsorbent may occur with time. A decrease in column capacity is an indication of this.

The photograph of the double-diffusion test in Fig. 5 shows that the group-

[1] The Group A antibody concentration in the serum before absorption was 1.18 mg/ml; in the absorbed serum it was 0.06 mg/ml. The Type 12 antibody concentration in the unabsorbed serum was 2.68 mg/ml and was 1.98 mg/ml in the absorbed serum.

In six unabsorbed Type 12 antisera, the range of concentration of anti-M antibodies was between 1.9 and 4.3 mg/ml, and that of anti–Group A antibodies was between 0.1 and 5.6 mg/ml.

specific antibody has been removed from a Type 1 anti-M serum by passage through the immunoabsorbent column. Two precipitin bands formed between the crude HCl extract well and the unabsorbed M1 antiserum well; one band was due to M protein, the other to Group A carbohydrate. The column-absorbed antiserum gave only an M type precipitin band with the M Type 1 HCl extract. A similar type-specific reaction is seen when M–Type 1 serum was used which had been absorbed in the usual way with heterologous streptococci. Group A serum is included in one of the wells to identify the group-specific band.

As much as 50 ml of serum has been successfully absorbed at one time with the column described above. If less than 10 ml of serum is to be absorbed by this procedure, the column volume should be reduced proportionately. The capacity of the immunoabsorbent is a guide to the size of the column which should be employed. 20 mg of Group A antibody can be absorbed by each milliliter of the immunoabsorbent employed here. Thus, a column with a 50 ml bed volume can theoretically absorb 1 g of Group A antibodies. For obvious reasons the amount of serum loaded on the column should contain considerably less antibody than the maximum capacity of the column if complete removal of Group A antibody is to be accomplished. For example, 50 ml of unabsorbed antiserum containing a total of 200 mg of Group antibody can be absorbed with the column employed above without any risk of overload.

Group A polysaccharide derived from Group A streptococci and purified by the formamide procedure has also been used to prepare an immunoabsorbent (14). After partial deacetylation this was coupled to cyanogen bromide–activated Sepharose and used on the column. While Group A antibodies can be absorbed with this immunoabsorbent, it was somewhat less effective than the one prepared from para-aminophenyl-*N*-acetylglucosamine.

Absorption of Anti-M Sera with an Immunoabsorbent Column for Capillary Precipitin Tests.—To circumvent a number of the technical problems which are associated with absorption with heterologous streptococci, the immunoabsorbent column has been employed here to absorb antisera for use in the capillary precipitin test. This is a particularly feasible approach when the cross-reactivity of a serum is primarily due to Group A antibodies, and for some sera passage through the column may be adequate to obtain sera sufficiently absorbed for type-specific precipitin reactions in the capillary precipitin test.

With other antisera, traces of antibodies to different cellular antigens remained in the column-absorbed sera and gave cross-reactions in the capillary precipitin test. If this was the case, these column-absorbed antisera were further absorbed using only a small volume of packed cells, 10 parts of serum to one part of packed streptococcal cells. These studies suggest that the Group A immunoabsorbent column warrants use as a routine for the preparation of M–type specific antisera for the capillary precipitin method as well as for the double-diffusion technique described here.

DISCUSSION

Although Michael and Massell (3) presented evidence that unabsorbed antisera and crude HCl extracts could be employed in the Ouchterlony technique for the M type classification of streptococci, a great deterrent to giving a suitable trial to the use of unabsorbed antisera was the possibility that cross-reactions would lead to erroneous results. It was, for example, hard to explain the discrepancy between these results, in which type identification was achieved with unabsorbed antisera, and the constantly observed common cross-reactions in the capillary precipitin tests due to the group-specific polysaccharide and unidentified protein antigens. The conservative method of using fully absorbed anti-M sera for identifying M types of Group A streptococci seemed essential until the time of the Michael and Massell report (3). The maintenance of the requisite panel of absorbed type-specific antisera consisting of more than 50 different M types for use in the capillary precipitin test is such a laborious and costly procedure that only a very limited supply is available. Furthermore, the absorbed sera do not always keep well. To facilitate epidemiological and clinical studies on streptococcal diseases, simpler methods which are also accurate for the identification of streptococci are imperative. For these reasons the use of unabsorbed M type antisera in the double-diffusion test has been reassessed and fully confirmed. Techniques have been developed for making this method of M typing fully reliable as a simple technique suitable for the M type identification of both laboratory and field strains of Group A streptococci.

In the Ouchterlony agar-gel technique, which employs crude extracts of streptococci and unabsorbed M type antisera, a precipitin line due to the M protein and another due to the group-specific carbohydrate are the two major reactions observed. The group-specific carbohydrate precipitin line is readily distinguishable from the M-protein line, even in extracts containing both antigens, because the group carbohydrate migrates more rapidly and to a greater distance from the extract well than does the M protein. There is a surprising lack of cross-reactive precipitin lines due to non–type-specific protein antigens in the extract. This degree of type specificity was observed when both standard stock strains and field strains were employed to prepare the acid extracts. Excellent agreement was observed between the M typing results obtained on 117 field strains by the conventional capillary precipitin method and the Ouchterlony double-diffusion method.

The lack of cross-reactions in the Ouchterlony technique can be explained by an examination of the properties of the agar gel as a supporting medium for the precipitin reactions. The relative concentrations of antigen and antibody, and differences in their diffusion rates through the gel, can determine the occurrence of visible precipitates in different antigen-antibody systems. Consequently, the minor cross-reactions due to antibodies in low concentration are eliminated by dilution of these antibodies as they diffuse from the central serum well. The cross-reactive "M-like" protein antigens diffusing slowly in

toward the center serum well from the peripheral extract wells do not meet sufficient concentrations of these cross-reactive antibodies to precipitate. Therefore, they do not often become apparent in the double-diffusion experiments with unabsorbed antisera and crude streptococcal extracts. In the capillary precipitin test, however, which has varying concentrations of these reactants present in different levels of the capillary tube, all antigen-antibody systems tend eventually to reach precipitable reaction mixtures except in inhibition zones of great excess of one of the reactants, especially of antigen. These suitable mixtures then give visible precipitates. The non–type-specific antibodies must be removed in order to obtain type-specific anti-M precipitin reactions. This is usually done by absorption with heterologous streptococci.

Although sera which contain Group A antibodies have been used successfully in these double-diffusion tests in the identification of field strains of streptococci, it is obvious that use of sera without Group A antibodies lessens the chance of misinterpretation and error. A survey of 465 unabsorbed antisera to 53 different serotypes in our stocks has revealed that 50% contain insignificant concentrations of antibody to the Group A carbohydrate. It is preferable to use these sera rather than those which contain large amounts of this antibody. If such antisera are not available, then Group A antibodies can be removed either by absorption in the usual way with packed heterologous streptococci, or by absorption with the immunoabsorbent column which has been introduced here. The immunoabsorbent first reported by Parker and Briles (4) is prepared by coupling para-aminophenyl-β-*N*-acetylglucosamine to Sepharose activated with cyanogen bromide. β-*N*-acetylglucosaminide is the immunodominant determinant of Group A cell wall polysaccharide (20). It is a highly effective absorbing agent for Group A group-specific antibodies.

Absorption with the specific immunoabsorbent column has a number of advantages over absorption with heterologous streptococcal cells, one of which is the shorter time needed to absorb the serum. A disadvantage of absorption with cells is that bacterial substances may go into solution.

In considering the history of type-specific M typing of Group A streptococci, it is notable that there has been a succession of methods employed. In 1918 unabsorbed sera were used in passive protection of mice to show the existence of specific types of hemolytic streptococci (21). The results were confirmed with specific macroscopic agglutination, also done with unabsorbed serum. This was followed by the discovery of the type-determinant substance, the M protein, with which parallel precipitin tests could be made (10). These were, in the first experiments, carried out with unabsorbed serum and M extracts from which the group polysaccharide had been removed by fractional precipitation with ethanol. In order to make the M precipitin test more useful so that crude HCl extracts could be employed in epidemiological work, the antisera were made type specific by absorption with streptococci of heterologous type (22). The type specificity of this precipitin reaction was confirmed by numerous immuno-

logical experiments and various non–type-specific reactions due to other antigens, e.g. T antigens and R antigens, were excluded. The capillary precipitin technique has been in use for 25 yr, and perhaps the double-diffusion technique will be substituted now if the results reported here are consistently confirmed in future trials.

SUMMARY

The successful classification of Group A streptococci by the capillary precipitin technique requires a complete series of M type antisera which are sufficiently potent and specific to give unequivocal type-specific reactions with all the serotypes. Specific antisera for this purpose have been prepared by absorption with heterologous streptococci.

Unabsorbed antisera have been employed here in the Ouchterlony double-diffusion agar-gel test to identify the M type of streptococci. Techniques have been developed for making this method of M typing fully reliable. The results reported here confirm and amplify the original findings of Michael and Massell (3). With crude HCl extracts and unabsorbed M type antisera, a precipitin line due to the M protein and another to the group-specific carbohydrate are the two major reactions observed. These reactions, however, are usually readily distinguishable. There was a surprising lack of cross-reactive precipitin lines due to non–type-specific protein antigens in the extracts.

Although many of the unabsorbed M type antisera can be employed in the double-diffusion tests, the group-specific antibody must be removed from some of the unabsorbed antisera to avoid confusing cross-reactions. Absorption of these antibodies has been achieved by means of a specific immunoabsorbent column prepared from para-aminophenyl-β-*N*-acetylglucosamine and cyanogen bromide–activated Sepharose. Excellent agreement was observed between the M typing results obtained on 117 field strains by the conventional capillary precipitin method and the Ouchterlony double-diffusion method.

APPENDIX

Method for Preparing Typing Antisera for Group A Streptococci for Use in the Capillary Precipitin Test

The preparation of M typing antisera for use in the double-diffusion test is the same as that described below except that the requirement for complete absorption of cross-reactions is less critical. This is discussed in detail in the text.

Preparation of Vaccine.—500 ml of culture (preferably grown in Todd-Hewitt broth) are centrifuged and resuspended in a small volume of physiological saline solution (0.85% NaCl) and killed by heating for 30 min in a 56°C water bath. The volume is brought to 60 ml and the vaccine is kept in the refrigerator.

Immunization Schedule.—Rabbits are given intravenous injections as follows, 0.5 ml on each of 3 successive days followed by 4 days rest in the 1st wk, and then 1.0 ml on each of 3 successive days followed by 4 days rest in the 2nd, 3rd, and 4th wk. On the 5th day after the last injection, a trial bleeding of 10–50 ml is taken from the

ear vein into a Vaseline-lined (Chesebrough-Ponds Inc., New York) tube. If the serum proves of satisfactory titer, the rabbit is bled to death the following day. If unsatisfactory, a further course is given of 3-ml injections followed after 5 days by a test bleeding.

If still unsatisfactory, two more weekly series may be given with test bleedings at the end of each series. If still unsatisfactory, rest periods of 1–2 months followed by similar weekly courses (three doses of 1 ml each) are given. Freshly prepared vaccines are used after the long rest periods.

Testing the Sera.—M extracts (2) are prepared from which the group carbohydrate, C, has been removed by precipitation of the M protein with 3 or 4 vol of ethanol, adding a few crystals of sodium acetate, until precipitin tests with the redissolved M substance are negative with potent Group A antiserum. A stock supply of such extracts of all type strains is kept for testing the crude antisera.

Absorption.—To ascertain which heterologous strains to use in absorbing the serum, capillary precipitin tests are set up with the column-absorbed serum to be tested and M extracts of all type strains. Those showing appreciable cross-reactions indicate the heterologous strains to use in absorbing the serum. The bacterial sediment from broth cultures of the strains indicated is taken up in a small amount of saline and heat-killed as above. The killed cultures are combined and well packed by centrifugation. About three parts of whole serum are used with one part of packed sediment. The mixture is incubated for 30 min at 37°C, then centrifuged at once, and the supernatant serum is tested for completeness of removal of group-specific and other cross-reacting antibodies. Only anti-M antibodies specific for the type under preparation are left.

Absorption is repeated with fresh culture if indicated by cross-precipitin reactions. One to three absorptions may be necessary. After absorption, the serum is filtered through a filter candle or Millipore filter, and Merthiolate (Eli Lilly & Co., Indianapolis, Ind.), 1:10,000 final concentration, is added.

Immunocolumn absorption as described in the text is also recommended to remove group antibodies, if present, as the first step, followed by absorption with bacterial sediment of heterologous strains as needed for residual cross-reacting antibodies.

Storage.—In The Rockefeller University laboratory, both absorbed and unabsorbed sera, after filtration, are stored in rubber-stoppered Pyrex containers at 4°C. Unabsorbed sera are stored without preservative and keep well for many years, often as long as 20 yr. Absorbed sera are less stable, but some lots keep well for several years. Capillary precipitin tests to check the homologous M reactions of absorbed sera should be carried out frequently.

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