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Purified Group A Streptococcal M-Protein in Latex Agglutination for detection of Type-Specific M-Antibodies

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Summary. M-proteins from several M-types of group A streptococci were extracted with hot acid and precipitated with 30% and 60% saturated ammonium sulphate giving two main fractions designated P60 and S60. After purification and serological testing the S60 fraction was found to be more specific and absorption of homologous sera with it removed their bactericidal activity. It was also used in latex agglutination tests to detect type-specific M antibodies in patients suffering from streptococcal sore throat, rheumatic fever, rheumatic heart disease and in normal individuals of comparable age groups and sex. The S60 latex agglutination test could also detect rise in antibody titre to the infecting streptococcal M-type.

Résumé. Proteins-M à partir des types-M du groupe A streptocoques furent extraites par l'acide chaud et précipitéee avec 30% et 60% de sulphate d'amoniaque saturé produisant deux fractions principales appelées P60 et S60. Après purification et expérience sérologique, on nota que la fraction S60 est plus spécifique et que son absorbtion de sérums homologues enlève à ceux-ci leur activité bactéricide. Cette méthode est aussi utilisée dans les expériences d'agglutination du latex pour détecter les anticorps M type-spécifique chez les malades souffrant de maux de gorge d'origine streptococcale, fièvre rhumatismale, troubles cardiaques d'origine rhumatismale, et chez les sujets normaux des mêmes age et sexe. L'expérience de l'agglutination du latex S60 montra aussi une augmentation en anticorps titre aux streptocoques du type-M.

INTRODUCTION

The detection and quantitative determination of antibodies to cellular antigens of group A β-haemolytic streptococci has recently attracted the attention of many workers (Molina & Saletti, 1961; Schmidt & Moore 1965; Karakawa, Osterland & Krause, 1965; Goldstein & Caravano 1967; Erwa, Maxted & Brighton, 1969, etc.). The main purpose of these efforts is to establish reliable techniques to help in the diagnosis of streptococcal infection and sequelae. In addition, such techniques could also help in the study of immunity and

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pathogenesis particularly the role of streptococcal antigens that cross-react with human heart and other human tissues (Kaplan, 1964; Zabriskie & Freimer, 1966; Nakhla & Glynn, 1967; Goldstein, Halpern & Robert, 1967).

However, most efforts have been directed to detect antibodies against streptococcal type-specific M-protein antigens, which are believed to be responsible for protection against infections with group A streptococci (Denny, Perry & Waunamaker, 1957; Lancefield, 1958, 1959; Dunlap & Harvy, 1967; Quinn & Lowry, 1967). Therefore, several methods for this purpose are available. The most important and most widely used is the bactericidal test first described by Todd (1927a & b). Originally, the test was a direct mixing of whole blood from convalescent patients with streptococcal culture, and after incubation reduction in number of streptococci was indicative of the presence of type-specific antibodies. Modifications have been introduced to detect M antibodies indirectly by mixing convalescent serum with blood from a non-immune person, followed by the streptococcal culture. The method used in this present investigation is the modification described by Maxted (1956). The test is highly specific and sensitive. Another reliable test is the long-chaining test (Stollerman & Ekstedt, 1957). This depends on the formation of long chains by group A streptococci in the presence of the homologous M antibodies. Mouse protection tests may also be used. These tests are biological tests and they are laborious and difficult to perform on a large scale. Moreover, the mouse protection test is expensive and extravagant in antisera.

There are also non-biological methods viz. hemagglutination tests (Fox, 1964; Vosti & Rantz, 1964; Quinn & Lowry, 1967), bentonite flocculation (Lian & Pierce, 1961), and lastly latex agglutination tests (Molina & Saletti, 1961; Bray *et al*, 1966; Brighton, 1967). For a specific reaction to be obtained in the latex agglutination test a pure antigen should be used for coating the latex particles. Therefore, in this report we will present results obtained consequent to an attempt made to prepare a reasonably pure, and serologically specific M-protein fraction. The aim is to achieve a reliable, specific and simple test for the detection and quantitative determination of M antibodies in patient's sera.

MATERIALS AND METHODS

Human sera

These were obtained from patients attending heart clinics and Ear, Nose & Throat outpatients in Khartoum and Omdurman Hospitals. Thus the following collections of patients' sera were available:

(1) Ten unpaired specimens of sera were obtained from patients suffering from uncomplicated rheumatic fever.

(2) Twenty-nine unpaired specimens of sera were collected from patients suffering from rheumatic heart disease.

(3) Twenty-one unpaired specimens of sera and six paired specimens were collected from twenty-seven patients suffering from uncomplicated sore throat.

In addition, sixty-five unpaired specimens of sera were collected from volunteers who were not suffering from streptococcal disease at the time of examination. This collection of sera was used as control. The ages of both patients and control groups ranged from 10 years to 35 years. Eighteen of the patients with sore throat were males and nine were females, in the uncomplicated rheumatic fever group seven were males and three were

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females and amongst the rheumatic heart patients twelve were males while seventeen were females. The control group consisted of forty-seven males and eighteen females.

Anti-sera prepared in rabbits

These were obtained from Dr W. R. Maxted, Streptococcal Reference Laboratory, Colindale, London. They included Lancefield group A specific anti-serum and type-specific M-protein antisera used in M-typing of group A β-haemolytic streptococci. Antisera for M-types 1, 2, 3M, 3R, 5, 6, 9, 12, 15, 18, 22 and 28 were used in this investigation.

Culture strains of group A streptococci

The following group A streptococcal strains isolated from patients and maintained in this Laboratory were used in this work:

M-type 1 : C.I.R. 249A
M-type 5 : C.I.R. 246A
M-type 6 : C.I.R. 271A
M-type 9 : R. 2748
M-type 12 : C.I.R. 274B
M-type 22 : R. 2088

All cultures were grown in Todd-Hewitt broth with added neopeptone and incubated in the air at 37°C. The broth was dispensed in 2-l bottles for obtaining mass cultures. For isolation of streptococci from patients, blood agar and crystal violet blood agar plates with bacitracin discs for preliminary identification of group A streptococci were used. Final identification of the organisms was confirmed by the Lancefield grouping (Lancefield, 1933).

Extraction of group-specific polysaccharide

Group-specific antigen was prepared according to the method of Fuller (1938) by hot formamide. It was purified by precipitation with acid alcohol and acetone as previously described (Erwa, *et al.* 1969).

Extraction and purification of the type-specific M-proteins

The hot acid extraction method (Lancefield, 1928) on intact cells from centrifuged 2-l amounts of Todd-Hewitt broth cultures of each streptococcal M-type was adopted. The deposited cells were resuspended in 15 ml N/2 HCL and boiled in a water-bath then neutralized by N/5 NaOH and the supernatant collected. Such extraction was repeated twice giving a total volume of crude extract of about 30 ml from each culture. Purification was carried out according to the method adopted by Erwa (1968) by the treatment of the crude extract with ammonium sulphate as follows:

(a) The crude extract was first precipitated in dialysis tubes suspended in 30% ammonium sulphate solution (30 ml saturated solution added to 70 ml distilled water with pH corrected to 7·2). The dialysis was effected at refrigerator temperature overnight. The precipitate formed was separated from the supernatant (designated S30) by centrifugation and discarded.

(b) A second precipitate was obtained when the S30 fraction was dialysed in 60% ammonium sulphate solution (60 ml saturated solution added to 40 ml distilled water, and pH corrected to 7·2). This precipitate (P60) was also separated from the supernatant (S60) by

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centrifugation. Ammonium sulphate was removed from both P60 and S60 fractions by dialysis against distilled water. To achieve complete dissolving of P60 fraction its pH was adjusted to 7·5. Removal of group-specific polysaccharide from P60 was effected by washing this fraction three times in four volumes absolute alcohol (Barkulis & Jones, 1957). The S60 fraction was purified by adjusting the pH to 5·5 to reprecipitate the M-protein (Lancefield & Perlmann, 1952), thus leaving the bulk of the group-specific polysaccharide in solution. The precipitate was redissolved and washed three times in absolute alcohol as before, with pH adjusted to 5·5, to get rid of traces of polysaccharide.

Chemical analysis

The chemical nature and purity of each fraction was investigated by the estimation of nitrogen and rhamnose contents as follows:

(1) *Estimation of nitrogen content:* This was done by a microKjeldahl technique employing a Markham still. The values were determined by a colorimetric estimation after nesslerization of the solution.

(2) *Estimation of rhamnose content:* Rhamnose was estimated by the cysteine-sulphuric acid reaction of Dische & Shettles as described by Dische (1955).

Serological tests

(1) Precipitin tests for group-specific polysaccharide were made by the precipitin ring technique of Lancefield (1933). An immediate reaction was graded as 4+, a reaction developing in the first minute as 3+, in the second minute as 2+, in 3–5 minutes as 1+ and after 5 minutes it was considered as negative.

(2) Capillary pipette precipitation tests for type-specific M-protein were made by the method of Swift, Wilson & Lancefield, (1943). A thick precipitate column rising to more than half of the height of the serum-antigen mixture was graded as 4+, $\frac{1}{2}$ of the height of the mixture was graded as 3+, $\frac{1}{4}$ of it was 2+, while $\frac{1}{8}$ was 1+. Less than 1+ was considered a trace reaction.

(3) Latex agglutination test was performed as previously described for the estimation of group A polysaccharide antibodies (Erwa *et al*, 1969). Suspensions of latex particles of the size 0·81μ were purchased from Difco. The test was performed in WHO agglutinating plates. The quality of agglutination was considerably improved by shaking the agglutinating plates by a mechanical horizontal rotatory shaker for 20–30 minutes at a speed of 125 rpm. The reading of the titres was made in the next morning after leaving the plates at room temperature.

(4) Absorption procedures followed were as follows:

(a) Purified M-protein extracts were added in equal parts to the neat serum and then incubated for 2–3 hrs. at 37°C. The precipitate was removed by centrifugation and the supernatant was used as initial serum dilution of 1:2.

(b) Latex suspensions coated with purified S60 fraction were dispensed in small test tubes in 2 ml quantities. Then they were centrifuged and supernatant discarded. The deposited particles were resuspended in 0·5 ml volumes of the sera to be absorbed, shaken and incubated at 37°C for 3–5 hrs. The latex particles were redeposited by centrifugation. The absorption was repeated twice with fresh particles. This technique was employed to absorb antibodies from human sera tested by S60 latex agglutination tests and by bactericidal tests for verification of results.

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(5) The indirect bactericidal test was performed according to the modified method described by Maxted (1956).

RESULTS

Results of tests on hyperimmune rabbit sera

After purification and freeing from polysaccharide, fractions P60 and S60 obtained from group A streptococcal types 1, 5, 6, 9, 12 & 22 were tested both chemically and serologically for the presence of type-specific M-protein. The results of all tests are depicted in Table I. Apparently the P60 fractions contain more protein per ml than the S60 fractions. Furthermore, the chemical analysis revealed complete absence of streptococcal polysaccharide in all purified fractions and this has been confirmed by the negative precipitin tests for group A specific reaction.

Serological tests for type-specific antibodies to types 1, 5, 6, 9, 12 and 22 group A streptococci were performed on homologous rabbit sera as well as heterologous rabbit sera as controls. Thus the controls used were as follows: type 3M & 3R anti-sera were controls for type 1 antiserum, type 12 anti-serum for type 5 anti-serum, type 2 for type 6, type 18 for type 9, type 15 for type 12 and type 28 for type 22. Each heterologous control antiserum was subjected to the same tests performed on the corresponding homologous antiserum. As Table 1 shows tests for M-protein precipitation reactions were positive for fractions P60 and S60 with relevant homologous antisera only; but no reactions were given with the corresponding heterologous antisera. Also it should be noticed that the precipitation reactions given by P60 fractions were generally stronger (2+ or 3+) than those given by the S60 fractions (Trace or only 1+).

However, both sets of fractions P60 & S60 were used in latex agglutination tests for M-protein antibodies. The results again are shown in Table 1. Latex suspensions prepared with S60 fractions gave much higher titres (up to 10,240) than suspensions with P60 fractions. In addition the latter suspensions gave some reactions with the corresponding heterologous anti-sera e.g. latex suspensions of P60 fraction for type 1 gave a titre of 640 with the homologous type 1 antisera and titres of 320 & 160 with the heterologous control type 3M and type 3R antisera respectively; also note titres for types 5 & 12 homologous and heterologous antisera. On the other hand no reaction was noticed in the case of latex suspensions of S60 fractions with the control sera.

The results of the indirect bactericidal tests performed on the same homologous and heterologous antisera absorbed by relevant P60 and S60 fractions showed that the S60 fractions removed antibodies responsible for the bactericidal activity of the homologous streptococcal type-specific M-antisera i.e. absorption of homologous antisera with S60 fractions of types 1, 5, 6, 9, 12 & 22 group A streptococci rendered these anti-sera without killing effect on the relevant streptococcal types. The P60 fractions showed no ability to remove the bactericidal activity from the anti-sera.

Results of tests on human sera

From the results obtained above the S60 fraction was obviously more specific than the P60 fraction. Therefore, for detection of type-specific streptococcal M antibodies the S60 fraction had been adopted as the antigen for use in latex agglutination. However, the indirect bactericidal test was repeated on five of the uncomplicated rheumatic patients' sera

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TABLE 1. Results of chemical analysis & serological tests on type-specific purified streptococcal M-protein fractions

M-protein fractions		Chemical analysis		Serological tests on hyperimmune rabbit sera						
		Protein content mg/ml.	Polysaccharide content ag/ml.	Group A specific reaction	Type-specific M-precipitation		Latex agglutination titres		Bactericidal activity	
					Homologous sera	Control sera	Homologous sera	Control sera	Absorbed antisera	Unabsorbed antisera
	P60	4·1	Nil	Negative	2+	—	640	3M:320, 3R:160	+	+
Type 1										
	S60	1·9	,,	,,	1+	—	1280	—	—	+
	P60	3·8	,,	,,	1+	—	320	Type 12: 160 Type 15: 160	+	+
Type 5										
	S60	2·6	,,	,,	1+	—	2560	—	—	+
	P60	3·7	,,	,,	3+	—	1280	Type 2: 40	+	+
Type 6										
	S60	2·1	,,	,,	1+	—	10240	—	—	+
	P6P	3·4	,,	,,	2+	—	160	Type 18: 40	+	+
Type 9										
	S60	1·3	,,	,,	Trace	—	640	—	—	+
	P60	3·9	,,	,,	2+	—	320	Type 5: 320 Type 15: 320	+	+
Type 12										
	S60	2·2	,,	,,	1+	—	2560	—	—	+
	P60	5·2	,,	,,	1+	—	160	Type 28: 20	+	+
Type 22										
	S60	1·4	,,	,,	Trace	—	5120	—	—	+

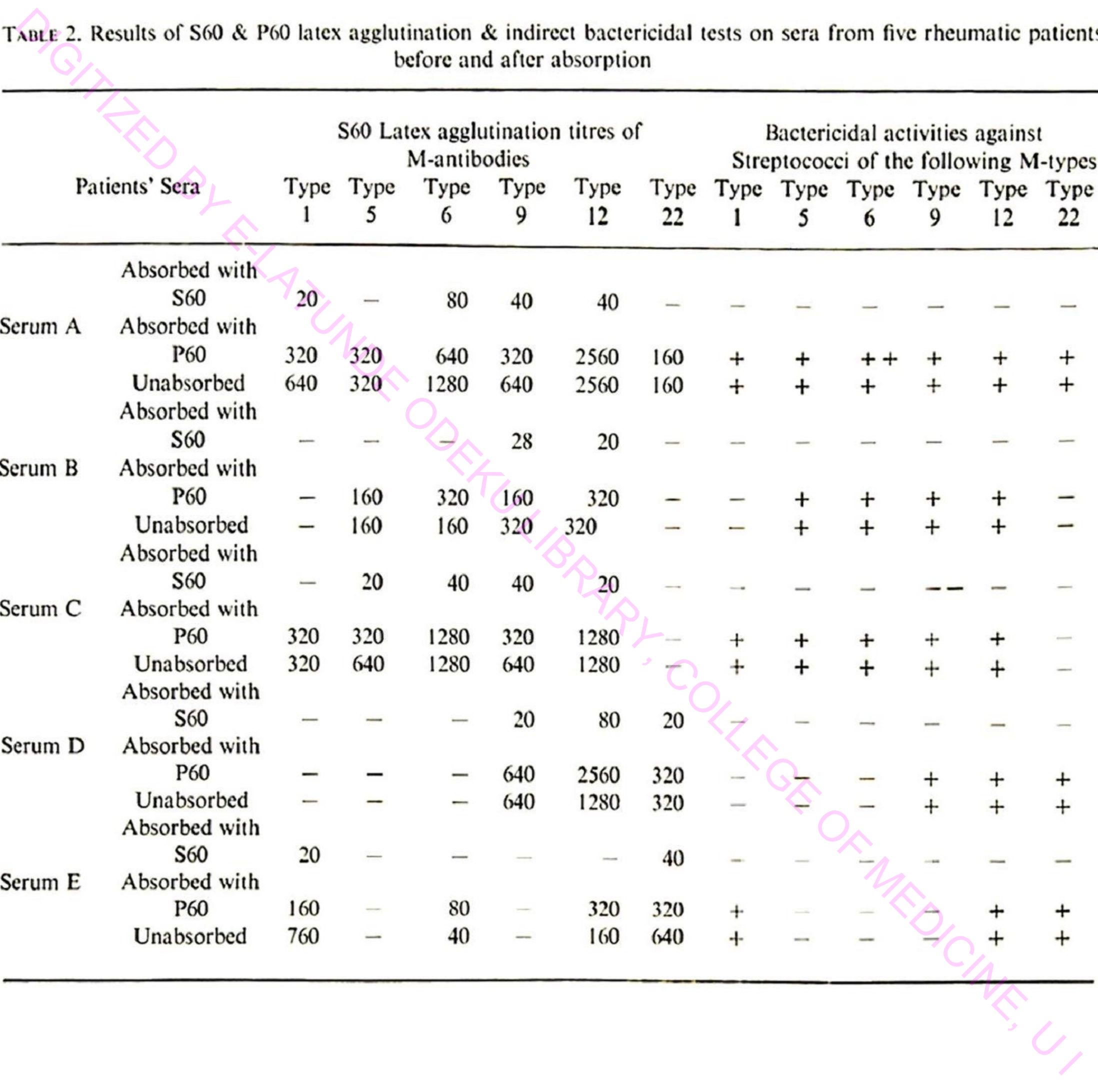

TABLE 2. Results of S60 & P60 latex agglutination & indirect bactericidal tests on sera from five rheumatic patients before and after absorption

Patients' Sera		S60 Latex agglutination titres of M-antibodies						Bactericidal activities against Streptococci of the following M-types					
		Type 1	Type 5	Type 6	Type 9	Type 12	Type 22	Type 1	Type 5	Type 6	Type 9	Type 12	Type 22
Serum A	Absorbed with S60	20	—	80	40	40	—	—	—	—	—	—	—
	Absorbed with P60	320	320	640	320	2560	160	+	+	++	+	+	+
	Unabsorbed	640	320	1280	640	2560	160	+	+	+	+	+	+
Serum B	Absorbed with S60	—	—	—	28	20	—	—	—	—	—	—	—
	Absorbed with P60	—	160	320	160	320	—	—	+	+	+	+	—
	Unabsorbed	—	160	160	320	320	—	—	+	+	+	+	—
Serum C	Absorbed with S60	—	20	40	40	20	—	—	—	—	— —	—	—
	Absorbed with P60	320	320	1280	320	1280	—	+	+	+	+	+	—
	Unabsorbed	320	640	1280	640	1280	—	+	+	+	+	+	—
Serum D	Absorbed with S60	—	—	—	20	80	20	—	—	—	—	—	—
	Absorbed with P60	—	—	—	640	2560	320	—	—	—	+	+	+
	Unabsorbed	—	—	—	640	1280	320	—	—	—	+	+	+
Serum E	Absorbed with S60	20	—	—	—	—	40	—	—	—	—	—	—
	Absorbed with P60	160	—	80	—	320	320	+	—	—	—	+	+
	Unabsorbed	760	—	40	—	160	640	+	—	—	—	+	+

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TABLE 3. Results of latex agglutination tests using S60 suspensions of streptococcal M types 1, 5, 6, 9, 12 & 22 on sera from patients and 65 controls

Streptococcal Disease	Number of patients			Number of patients giving titres of 160 and over with S60 latex suspensions of M type streptococci.					
	Male	Female	Total	Type 1	Type 5	Type 6	Type 9	Type 12	Type 22
Uncomplicated sore throat	14	7	21	12 (57·1%)	16 (76·1%)	11 (52·4%)	14 (66·7%)	17 (81·0%)	9 (42·9%)
Uncomplicated rheumatic fever	7	3	10	4 (40%)	9 (90%)	7 (70%)	8 (80%)	9 (90%)	6 (60%)
Rheumatic heart disease	12	17	29	19 (65·5%)	26 (89·7%)	17 (58·6%)	23 (79·3%)	25 (86·2%)	17 (58·7%)
Normal individuals (controls)	47	18	65	31 (47·7%)	37 (57·0%)	19 (29·2%)	25 (38·5%)	47 (72·3%)	23 (35·4%)

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containing streptococcal M antibodies, to reconfirm the ability of the S60 fractions to remove their killing effect on the relevant streptococcal cultures. The results are shown on Table 2. From these results it is obvious that each serum contains antibodies to more than one M type streptococci. The bactericidal activity was effectively removed from all five sera after absorption of each of the M antibodies with the homologous streptococcal S60 fraction (the absorption was carried out by latex suspensions of S60 fractions as described in Materials and Methods.). The same sera were also tested after absorption with P60 latex suspensions in the same way, but no removal of bactericidal effect was noticed.

Following these verification tests latex suspensions were prepared with S60 fractions of each of the six streptococcal M types used in this investigation. Each serum from 21 patients suffering from uncomplicated sore throat, ten patients suffering from uncomplicated rheumatic fever, twenty-nine patients suffering from rheumatic heart disease and sixty-five normal individuals (controls) was tested with the six S60 latex suspensions individually. M antibody titres of each type in the sera were determined. It was found that each serum reacted to more than one type-specific S60 suspensions in the same manner as had happened in the five selected sera shown in Table 2. The collective results of all patients and control sera are depicted in Table 3. Considering high titres of 160 and over it is clear that patients with streptococcal disease showed much higher M antibody titres than the control individuals. Type 5 & type 12 antibodies are by far the commonest (75–90%). Type 9 is next in frequency (66%–80%) followed by type 6, 1 & 22 in that order. In the control individuals type 12 antibodies are the most predominant (72·3%) followed by type 5 (57%). Other antibodies are much less frequent especially when compared with those of the patients.

The paired sera from the six patients with uncomplicated sore throat were tested separately. The infecting group A streptococcal type from each patient was isolated from a throat swab after inoculation on blood agar. Three patients were found to be infected with group A M-type 9 streptococci, one with M-type 5, another with M-type 12 and the last with M-type 22. After agglutination tests with the S60 latex suspensions were performed

TABLE 4. Results of S60 latex agglutination tests on six pairs of sera from six patients with sore throat

Patient	Infecting streptococcal M-type	Serum	Titres of M-antibodies Type 1	Type 5	Type 6	Type 9	Type 12	Type 22
M.O.K. (Male)	5	1st	—	—	160	40	320	—
12 years		2nd	—	320	160	40	320	—
H.I.H. (Male)	9	1st	160	80	—	160	160	40
15 years		2nd	80	80	—	640	160	40
Z.A.M. (F)	9	1st	—	160	40	40	80	320
22 years		2nd	—	320	80	1280	160	320
B.A.M. (F)	9	1st	320	—	160	—	40	80
30 years		2nd	320	—	160	160	40	80
B.H.Y. (Male)	12	1st	—	40	—	160	20	—
26 years		2nd	—	80	—	160	640	—
E.H.I. (Male)	22	1st	—	320	80	320	40	—
14 years		2nd	—	640	80	160	20	160

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each serum gave high M antibody titres to more than one M-type in addition to the infecting type (Table 4). Moreover, the titre of the second serum showed a rise of four folds or more for M antibodies against the infecting atreptococcal type in each patient.

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DISCUSSION

The above results have demonstrated that type-specific M-proteins of group A streptococci contain at least two serologically active fractions viz. the P60 and the S60 fractions. The latter has been found to be more specific than the former. The specificity of the reactions involving the S60 fractions has been verified by their ability to remove bactericidal activity from both hyperimmune rabbit sera and patients' sera containing the relevant M-antibodies. Accordingly the S60 fractions are favourable and reliable representatives of M-proteins for accurate detection of type-specific antibodies. A similar M-protein fraction having serological specificity and bactericidal activity was described in type 17 group A streptococci by Wilson & Wiley (1963).

The streptococcal M-proteins studied here gave appreciable cross-reactions only in the case of P60 with most heterologous antisera. However, cross-reactions may also be given by a few S60 fractions (Erwa, 1968) e.g. cross-reactions between type 14 and type 49 which are known to be closely related group A streptococcal types. Moreover, Hirst & Lancefield (1939) reported cross-reactions between M-types during their study on passive immunity by mouse protection tests, and thus they demonstrated cross-immunity between certain types. In our experience cross-reactions in the case of S60 fractions are relatively uncommon.

The human sera from patients with various streptococcal disease contained antibody to several M-types of group A streptococci when tested by the S60 latex agglutination test and the indirect bactericidal test. This is in agreement with results obtained by Markowitz (1963) and Stoyanova (1966) who both used the indirect bactericidal test. Moreover, rise in the titre was shown in M antibodies in the second sera of the paired sera to the infecting streptococcus.

In conclusion the S60 fractions are reasonably pure antigens conforming with the criteria for the basic characteristics of the type-specific M-proteins of group A streptococci, viz. specific serological reaction and anti-bactericidal activity. However, the value of the S60 fractions in the production of protective antibodies by immunisation of experimental animals has not yet been assessed.

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