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An M-associated protein antigen (MAP) of group A streptococci

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SUMMARY

A streptococcal antigen that is closely associated with the M-antigen, but is not type specific can be detected by means of a complement-fixation test in extracts of M-positive, but not of M-negative, variants of group A streptococci. Purification of acid extracts results in a concomitant increase in the purity both of the type-specific M-antigen and of the M-associated protein (MAP). Antibody to MAP is present in the sera of patients who have had streptococcal infection. The highest titres are found in patients with rheumatic fever.

INTRODUCTION

Group A streptococci that synthesize a type-specific M-antigen will multiply in normal human blood. If the corresponding antibody is added to the blood the streptococci are opsonized and killed by phagocytosis (Todd, 1927). The addition of extracted M-protein of the homologous type will neutralize the antibody and prevent this bactericidal action. During investigations into the M-protein content of fractions obtained from group A streptococci, we observed that, in the blood of some donors, the bactericidal action on M-positive streptococci in the presence of homologous rabbit antiserum was also neutralized by extracts of streptococci of heterologous M-types.

MATERIALS AND METHODS

Strains

Group A streptococci were stock laboratory cultures and cultures that had been submitted to the Streptococcus Reference Laboratory for routine typing. Members of other groups were stock cultures. Paired M-positive and M-negative variants of various types were also available (see Widdowson, Maxted & Grant, 1970).

Medium

The growth medium was Oxoid Todd-Hewitt Broth (Oxoid Ltd., London) with the addition of 1% (w/v) Neopeptone (Difco Ltd., Detroit, Michigan, U.S.A.).

Rabbit sera

M-antisera against whole heat-killed streptococcal cells were prepared in the Streptococcus Reference Laboratory.

Human sera

Sera from cases of rheumatic fever, glomerulonephritis and uncomplicated streptococcal infection were obtained from hospitals and Public Health Laboratories. 'Normal' sera had been submitted for Wassermann tests or were from laboratory workers.

The bactericidal test

This was carried out as described by Maxted & Valkenburg (1969). In tests in which streptococcal extracts were added to the bactericidal system 0·02 ml. quantities of extract were added to 0·02 ml. of M-antisera prepared in rabbits; 0·02 ml. of a suitable dilution of a 3–4 hr. culture of streptococci of a homologous type (containing 50–200 colony-forming units per 0·02 ml.) was added to the mixture in small glass tubes; 0·3 ml. of heparinized human blood was added to each tube, and the tubes were sealed and incubated at 37° C. for 3 hr. in a rotating drum. The tubes were opened, and 0·02 ml. quantities of the reaction mixture were inoculated into pour plates of Hartley digest agar (Cruickshank, 1965) together with 10% (v/v) horse blood. After 24 hr. at 37° C. the plates were examined and survival was scored as follows: + + + + = confluent growth, + + + = > 200 colonies, + + = 50–200 colonies, + = up to 50 colonies and − = no colonies.

The complement-fixation test (CFT)

This was performed as described by Bradstreet & Taylor (1962). Various streptococcal fractions were used as antigen, and the complement dose was three times the minimum haemolytic dose (3 MHD). Fixation was allowed to take place overnight at 4° C. After the addition of the sensitized sheep red cells the tests were incubated at 37° C. for 30 min. The results were recorded after 2 hr. at 4° C. as + + + + = no haemolysis, + + + = slight haemolysis, + + = 50% haemolysis, + = about 75% haemolysis, 0 = complete haemolysis.

Many human sera tested were anti-complementary in preliminary tests. This was eliminated by incubation of the serum with 3 MHD of complement at 37° C. for 30 min. The sera were then inactivated at 56° C. for 30 min.

Preparation of streptococcal extracts

Hot-acid extracts were made with 0·2 N HCl by the method of Lancefield (1928) and formamide extracts by the method of Fuller (1938). Purified M-antigens were prepared from crude 0·2 N acid extracts by precipitation at pH 2, ribonuclease treatment and ammonium sulphate fractionation (Lancefield & Perlmann, 1952), carboxymethyl cellulose chromatography (Fox & Wittner, 1965) and polyacrylamide gel electrophoresis (Widdowson, Maxted, Grant & Pinney, 1971). M-antigens of various types were purified, and at each stage of purification the

volume of the fraction was recorded and the protein content per ml. was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). The complement-fixation titre at each stage was determined by titration of twofold dilutions of the fraction with suitable dilutions of the test human serum. The titre of type-specific M-protein of each fraction was determined by a gel-diffusion test of twofold dilutions of the fraction against appropriate absorbed specific M-antiserum.

Absorption of human sera

All absorptions were carried out at 4° C. for 18 hr. For absorption with whole streptococcal cells, overnight broth cultures were centrifuged and the cells washed three times in 0·85% (w/v) saline. The packed cells (1 cm. depth in $3 \times \frac{1}{2}$ in. test tubes) were used to absorb 1 ml. of the serum diluted 1/10 in phosphate-buffered saline pH 7·5. Membranes of a type 49 M-positive strain were prepared by the phage–lysin method of Freimer (1966). Washed membranes were suspended in saline and the optical density was adjusted to 1·5 at 600 nm. in a Unicam SP 600 Spectrophotometer. This suspension was mixed with an equal volume (1 ml.) of 1/10 dilutions of the sera under test. Samples of normal human heart and skeletal muscle and rabbit heart, kidney and skeletal muscle were finely minced and washed in normal saline until the washings were clear. The minced tissue was strained through muslin and used (1 cm. depth of packed tissue in $3 \times \frac{1}{2}$ in. test tubes) to absorb 1 ml. quantities of 1/10 dilutions of serum.

Anti-streptolysin O tests

These were performed by the method of Gooder & Williams (1959).

Streptococcal anti-DNAse B tests

These were done by the method of Nelson, Ayoub & Wannamaker (1968).

RESULTS

Addition of extracted M-antigen to the bactericidal system

The effect of adding crude acid extracts of group A streptococci to the indirect bactericidal system (M-positive streptococcus + homologous rabbit antiserum + fresh human blood) is summarized in Table 1. The expected result was obtained with the blood of 8 out of 10 normal donors; only the addition of extracted M-antigen of the homologous type resulted in the survival of the streptococcus. However, the blood of two donors, who had no history of recent streptococcal infection, gave a different result; the addition of acid extracts of M-positive, but not of M-negative, streptococci of any type abolished the bactericidal effect. Table 1 also shows the results of addition of acid extracts of M-positive and M-negative variants to the direct bactericidal system, where the survival of an M-negative streptococcus, usually unable to multiply in human blood, was promoted in the presence of M-positive extracts, but again only in the blood of the same two abnormal donors. Formamide extracts, which contain the group carbohydrate but no M-antigen, had no effect on either system.

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Although there is evidence that complement is involved in the phagocytosis of M-negative streptococci, the participation of complement in the phagocytosis of M-positive cells opsonized by type-specific antibody has been questioned (Stollerman, Rytel & Ortiz, 1963; Stollerman, Alberti & Plemmons, 1967; Saito, 1970). However, the requirement for small amounts of complement, in addition to the heat-labile opsonins described by Stollerman *et al.* (1963), cannot be ruled out, and we considered that the observed reversal of the bactericidal effect in both the direct and indirect systems might be explained on the basis of complement depletion by an extraneous antigen–antibody system, and that the antigen involved was associated with M-antigen.

Table 1. *The effect of addition of extracted M-antigen in the bactericidal system*

Present in all tubes	Additions: Rabbit antiserum	Additions: Extract	Amount of growth after 3 hr. at 37° C.*: Expected result	Amount of growth after 3 hr. at 37° C.*: Result with blood of certain donors
Human blood + M-positive group A streptococcus	Nil	Nil	+ + + +	+ + + +
	Homologous anti-M serum	Nil	−	−
	Homologous anti-M serum	Acid extract of homologous M type	+ + + +	+ + + +
	Homologous anti-M serum	Acid extract of any heterologous M type	−	+ + + +
	Homologous anti-M serum	Acid extracts of M-negative variants	−	−
	Homologous anti-M serum	Formamide extract of M-positive streptococcus	−	−
Human blood + M-negative group A streptococcus	Nil	Nil	−	−
	Nil	Acid extract of any M-positive streptococcus	−	+ + + +
	Nil	Acid extracts of M-negative variants	−	−
	Nil	Formamide extract of M-positive streptococcus	−	−

* 0·02 ml. of reaction mixture was inoculated into pour plates of Hartley digest agar + 5 % (v/v) horse blood; + + + + = confluent growth; − = no growth (see text).

Detection of M-associated antigen by complement-fixation tests with sera of abnormal donors

To test the hypothesis that the sera of the abnormal donors contained an antibody that fixed complement in the presence of streptococcal extracts, we performed complement-fixation tests on these sera with 0·2 N acid extracts of M-positive streptococci of 50 different types, and with M-negative variants obtained from streptococci of 10 different types. The sera of both the abnormal donors fixed complement (one at a dilution of 1/40 and the other at 1/20) in the presence of a 1/50 dilution of all the Lancefield extracts of M-positive streptococci, but not in the presence of

the extracts of M-negative streptococci. No fixation was obtained with the sera of 'normal' donors at a dilution of 1/4 even when tested with undiluted extracts of the streptococci. Titration of acid extracts of M-positive strains in complement-fixation tests with a fixed dilution of 'abnormal' serum gave titres of at least 80, and often as high as 320 or 640. In general, a high CFT titre was associated with a high titre for type-specific M-antigen in the extract. Fixation by M-negative extracts was not observed at dilutions higher than 1/5. Formamide extracts of M-positive variants did not fix complement in the presence of the serum of abnormal donors.

Lancefield extracts of representative strains of streptococci of groups B to R were tested for complement fixation with a suitable dilution of the serum of an abnormal donor. In general, complement was not fixed with extracts of streptococci of groups other than A. The exceptions were certain group C and G strains that were capable of survival in human blood, and thus might be assumed to have M-like antigens. Extracts of group G strains known to have type 12 M-antigen (Maxted & Potter, 1967) also contained the complement-fixing antigen.

Sensitivity of the M-associated antigen to proteolytic enzymes

The ability of extracts of M-positive strains to precipitate with their type-specific antiserum, to fix complement with the sera of abnormal donors, or to produce an apparent non-specific neutralization effect in the bactericidal test with the blood of these donors, was abolished by treatment at 37° C. with trypsin, pH 7·8, in 15 min., with pepsin, pH 5·2, in 15 min., and with streptococcal proteinase (Elliott, 1945) in 2 hr.

Purification of M-associated protein antigen (MAP)

Crude acid extracts of strains of M-type 5 and M-type 30 were prepared and purified by precipitation at pH 2·0, ribonuclease treatment, ammonium sulphate fractionation and carboxymethyl cellulose chromatography.

The protein content per ml., the CFT titre and the titre of type-specific M-protein were determined at each stage. Table 2 shows the results obtained with the type 5 strain. The minimum quantity of protein required to give a positive reaction in tests for both antigens was determined at each stage by dividing the protein content of the extract by the titre of the reactions, and this was used as a measure of the purification of the antigens. About 10 times more protein was required to give a positive precipitin line by gel diffusion with the homologous M-antiserum than to give a positive CFT with the serum of an abnormal donor. The purity of the two antigens was increased in almost identical ratio (1 to 7·3–7·4) by precipitation with 60% saturated ammonium sulphate, and by subsequent fractionation of this precipitate on carboxymethyl cellulose (1 to 26·7). The results of fractionation of the P 60 fraction (i.e. the precipitate obtained between 33% and 60% saturation with ammonium sulphate) on carboxymethyl cellulose with a pH gradient from 4·0 to 7·0 showed that the material in the second peak (pH 7·0) had higher activities for both proteins than the first peak (pH 5·5) which contains proteins other than M-protein and probably residual nucleic acid (Fox & Wittner, 1965).

Table 2. *Purification of M-antigen and MAP from a crude Lancefield extract of a type 5 strain*

Fraction	Volume (ml.)	Protein (mg./ml.)	*M-antigen titre	†CFT titre	Minimum quantity (μg./ml.) of protein required for a positive precipitin test‡	Minimum quantity (μg./ml.) of protein required for a positive CFT‡	Purification factor for M-antigen and MAP§
Crude extract	260	2·55	8	64	320	40	1·0
pH 2 precipitate	22	6·10	32	256	190	24	1·7
pH 2 supernatant	233	2·1	0	0	—	—	—
33 % $(NH_4)_2SO_4$ precipitate	14	0·425	0	8	—	53	< 1
60 % $(NH_4)_2SO_4$ supernatant	39	0·575	1	16	575	36	< 1
60 % $(NH_4)_2SO_4$ precipitate	25	2·775	64	512	43	5·5	7·3–7·4
CMC 1st peak (pH 5·5)	—	0·630	8	64	76·5	10	4·0–4·2
CMC 2nd peak (pH 7·0)	—	0·390	32	256	12	1·5	26·7

* Highest twofold dilution to give a precipitin line in gel diffusion test with absorbed anti-M serum.
† Highest two-fold dilution to give a 2+ fixation in the CFT with a fixed dilution of an anti-MAP serum.
‡ Protein content/titre.
§ Calculated relative to crude extract.

Purification of the P 60 fraction of an M-type 30 strain by CMC chromatography showed that, as in the case of the type 5 strain, the second peak had higher specific activity than the first peak for both type-specific M-protein and MAP. Minimum quantities of protein to give a positive CFT for type 30 M-protein were: 5 μg./ml. of P 60 fraction, 5–10 μg./ml. – first peak, and 1–1·5 μg./ml.–second peak.

M-proteins of three other types (12, 24 and 49) were purified as far as the P 60 stage. Complement-fixation tests on these P 60 fractions showed that, as with the corresponding fractions of type 5 and type 30 M-protein, approximately 5 μg./ml. of protein was required to fix complement in each case.

Polyacrylamide gel electrophoresis of a P 60 fraction of a type 24 M-protein showed one main band and several minor ones. Both type-specific M-protein and MAP could be detected most strongly in the main band. The minor bands also contained both antigens.

The presence of anti-MAP in human sera

Twofold dilutions of sera from patients with various streptococcal disease were tested for complement fixation with a suitable dilution (determined by preliminary titration) of the purified P 60 fraction of M-protein from a type 30 strain. This type is rare in the U.K. and was chosen to avoid possible complement fixation by a type-specific antigen–antibody reaction. In some experiments, crude acid extracts

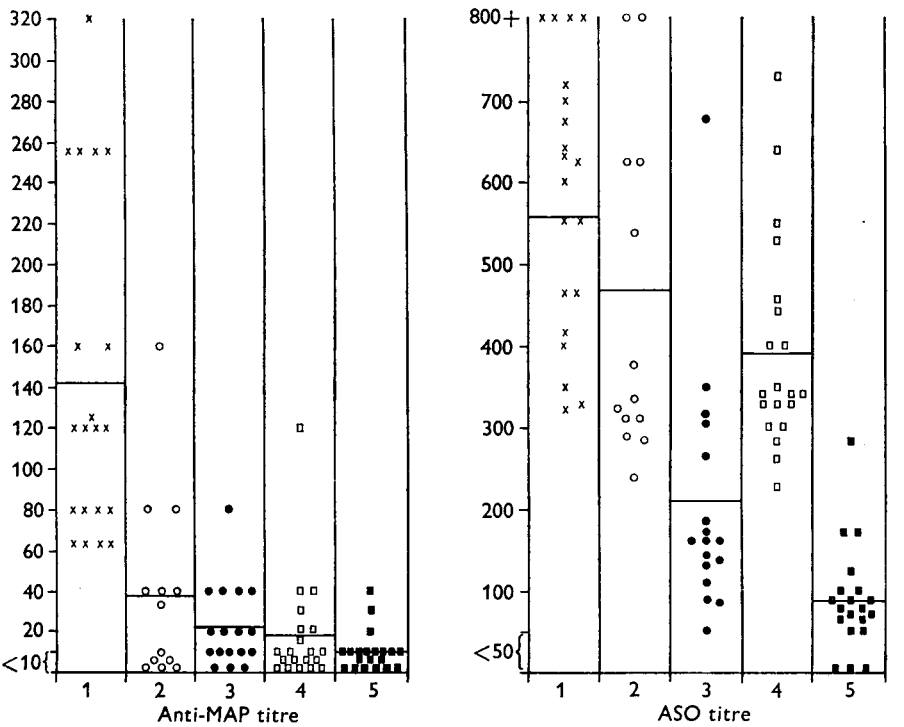

Fig. 1. Titres of anti-MAP and ASO in streptococcal diseases × *(1) Rheumatic fever.* ○ (2) Nephritis following throat infection. ● (3) Nephritis following skin infection. □ (4) Uncomplicated streptococcal infection (throat). ■ (5) Normals.

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of various types were also used as antigen. Such extracts contain many impurities, and fixation due to antibodies to these seemed a possibility. However, in tests with human sera and crude acid extracts of M-positive and M-negative strains, and with formamide extracts, fixation occurred only with the extracts of M-positive strains, and the titre of the reaction was identical with that obtained with purified M-proteins whatever the M-type of the strain used. This suggests that the purity of the M-antigen may not be an important criterion in complement-fixation tests with human sera.

Fig. 1 shows the scatter of anti-MAP titres in various human sera. The arithmetical mean for each category is also shown.

The titre exceeded 60 in all of the 20 cases of rheumatic fever tested but only in 4 of 30 cases of acute nephritis and 1 of 30 cases of uncomplicated streptococcal infection. The average titre in rheumatic fever was 140, about 4 times higher than that of the other categories of infection considered together. The anti-MAP titre in 'normal' individuals was generally not greater than 10.

Although many of the patients with rheumatic fever had high ASO titres some did not, and the average titre in rheumatic fever was not greatly different from that seen in nephritis following throat infection and in uncomplicated streptococcal disease. In nephritis secondary to streptococcal skin sepsis, on the other hand, the ASO titre was seldom significantly raised (Potter *et al.* 1968; Anthony, Perlman & Wannamaker, 1967; Dillon & Reeves, 1969).

These results are summarized in Table 3, which also gives the anti-DNAse B titres for the cases of nephritis following skin infection. These confirmed that the patients had indeed suffered from a recent streptococcal infection (Kaplan *et al.* 1970). There is little doubt, therefore, that the anti-MAP response in nephritis is normally lower than that in rheumatic fever.

Removal of antibody to M-associated protein by absorption

The relative titres of anti-MAP in sera from patients with rheumatic fever and with the other streptococcal diseases are similar to the relative titres of heart-reactive antibody in the sera of comparable groups of patients (Zabriskie, Hsu & Seegal, 1970). The presence of heart-reactive antibodies in human and rabbit sera can be demonstrated by fluorescent staining of human or rabbit heart sections, and Zabriskie & Freimer (1966) and Kaplan (1966) have shown that the antibodies are absorbed by whole streptococcal cells, cell walls and protoplast membranes of group A streptococci. There is, however, considerable disagreement as to the situation of the cross-reactive antigen in the bacterial cell or whether or not there is more than one cross-reactive antigen (Zabriskie, 1969; Kaplan, 1969). Human sera with high anti-MAP titres were absorbed with whole cells of M-positive streptococci (types 5, 24 and 30), with the protoplast membranes of a type 49 M-positive strain and also with the homogenates of mammalian tissues reported to bind heart-reactive antibody. These included human heart and skeletal muscle and rabbit heart and skeletal muscle. Complement-fixation tests on sera absorbed in this way showed that anti-MAP had been completely removed from the serum. Absorption with *Staphylococcus aureus* did not absorb the antibody. Absorption

Table 3. *Titre of antibodies to three streptococcal antigens in sera from patients with various streptococcal diseases*

Diagnosis	Number of cases	ASO titre		Anti-DNAse B titre		Anti-MAP titre*	
		Average	Range	Average	Range	Average	Range
Rheumatic fever	20	560	320–984	—	—	143	64–320
Nephritis (following throat infection)	13	473	235–1128	—	—	38	0–160
Nephritis (following skin infection)	17	209	50–675	5100	640–12800	21·25	0–80
Uncomplicated streptococcal infection	20	389	230–730	—	—	17·7	0–120
Normals	20	88	< 50–280	144	< 50–400	9·8	0–40

* Titres of < 10 recorded as 0.

with rabbit kidney gave rather variable results which in general showed a reduction in CFT titre rather than complete removal of the antibody. However, these preliminary experiments were done with relatively large amounts of absorbing agents, and no quantitative assessment has yet been made of the minimum relative quantities of streptococcal cell walls and purified cell membranes needed to remove anti-MAP from the sera (Zabriskie, 1969).

DISCUSSION

The results strongly suggest that the complement-fixing antigen which is present in all M-positive streptococci is closely associated with M-antigen itself. The concomitant increase of specific activity of the two antigens at each stage of the process of purification indicates that they may form part of the same molecule or complex. The existence of a non-specific protein inseparable from M-antigen has previously been suggested by Johnson & Vosti (1968) to explain the cross-reactions observed with some sera when haemagglutination tests with tanned red cells coated with purified M-antigen were used to detect anti-M antibodies in human and rabbit sera. Similar cross-reactions were also observed by Erwa (1968), who used latex particles coated with purified M-antigens. These findings could be explained, in part at least, by the presence of an MAP component of the M-antigen preparations used. Anthony (1970) showed that, in sensitive methods for the quantitative measurement of M-antibody such as the ^{131}I-labelled antigen technique, the most serious limitation is the purity of the antigen. In a study with ^{131}I-labelled type 12 M-protein, purified by ammonium sulphate fractionation and CMC chromatography, he found that the reaction of the antigen with heterologous rabbit sera could be prevented only by the addition of a purified antigen of a heterologous type. Although such cross-reactions are often attributed to insufficient purification of the antigen, our results indicate that increased purification of the type-specific antigen simply resulted in similar purification of the potentially cross-reactive element, MAP. A similar method of purification was used by Beachey, Alberti & Stollerman (1969) in studies of hypersensitivity in the guinea-pig; in these, increasing purity of the M-antigen often resulted in an increased cross-reactivity between M-types.

We have begun to investigate the presence of antibodies to MAP in rabbit antisera. The multiplicity of antibodies to other components of the streptococcal cell, and the presence of anti-complementary activity that is sometimes not removable by pretreatment with complement, makes interpretation of these results difficult. It does appear, however, that antibodies to MAP are present in some but by no means all hyperimmune rabbit sera. Titres up to 320 have been observed.

The ability to detect MAP in group A streptococci by means of a complement-fixation test with an appropriate human serum is useful in streptococcal typing. Many strains, particularly those isolated from skin lesions, cannot be M-typed with the antisera at present available. To find out if they are M-positive has hitherto required the performance of a direct bactericidal test in the blood of at least three normal donors. We find that acid extracts of all apparently untypable strains that are capable of survival in normal human blood will fix complement in the

presence of a human serum that contains anti-MAP. Moreover, the highest dilution of the extract at which complement is fixed with a standard dose of serum gives a good indication of the amount of MAP, and hence the amount of M-antigen, produced by the strain. This could prove useful in selecting a suitable vaccine strain for the production of type-specific antiserum against M-antigens of new types.

The highest titres of antibody to MAP were found in patients with streptococcal infection who had rheumatic fever. The relative titres found in rheumatic fever compared to those seen in sera from other types of streptococcal disease are similar to those described by Zabriskie *et al.* (1970) in their fluorescent-antibody studies of heart-reactive antibody. These workers believe that the streptococcal antigen that is cross-reactive with heart tissue is not M-associated but is present in the streptococcal cell membrane of all group A strains. On the other hand Kaplan (1966, 1969) suggests that there are at least two cross-reactive streptococcal antigens and favours a close association of one of these (CR 1) with M-antigen.

Our preliminary absorption experiments also suggest a similarity of anti-MAP to heart-reactive antibody. Should more carefully controlled quantitative experiments confirm a relationship, then the undoubted association of the MAP antigen with M protein may prove to have some bearing on the divergent views concerning heart-reactive antibody.

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