TYPE-SPECIFIC ANTIGENS, M AND T, OF MATT AND GLOSSY VARIANTS OF GROUP A HEMOLYTIC STREPTOCOCCI*

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The immunological reactions of a newly identified part of the type-specific antigenic complex of group A hemolytic streptococci will be described in this paper. The relationship of this substance, designated as T, to the previously described type-specific M substance and its bearing on the type-specific reactions of matt and glossy variants of members of this group of streptococci will be discussed, but a report of the chemical characteristics of the new antigen must await further investigation.

Hemolytic streptococci have long been known to exhibit type specificity[1] demonstrable by agglutination and precipitin reactions and by passive protection tests in mice (1). The substance, M, apparently responsible for these reactions has been separated from the bacterial cells in partially purified form and found to be protein in nature (2). Originally, this substance was considered a hapten, which reacted with antibody in type-specific immune serum, but no antigenicity was demonstrable after it was extracted from the cell. Later, however, fractions of somewhat similar nature were prepared in antigenic form (3). More recently, it was also shown that the type-specific M substance as originally prepared was somewhat antigenic if used in large enough dosage, although less active than more gently treated material (3 *e*).

This type-specific substance, M, is characteristic of the matt form[2] of group A hemolytic streptococcus (4) but is absent, or present in only minute amounts, in degraded glossy variants. The latter variants sometimes arise spontaneously in cultures on artificial media and may also be obtained by serial subculture of the matt strain in homologous type-specific immune serum. In the matt phase in which group A hemolytic strepto-

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[1] These strains have since been identified as members of the serological group A.

[2] The matt variant studied here probably corresponds to the "mucoid" of Dawson's system of nomenclature, and the glossy variant to his "smooth" (5).

cocci are isolated from human infections, these organisms are occasionally highly virulent for mice or may become so with relative ease on repeated passage through mice; but the glossy derivative is much less virulent and is converted into the virulent form with much more difficulty and only after the restoration of its type-specific M substance and the accompanying properties dependent on this substance (4).

In spite of the fact that the glossy variant appears to be almost entirely devoid of type-specific M substance, this degraded form still exhibits one strikingly type-specific feature: it agglutinates type specifically in immune serum containing the homologous type-specific antibody, and on injection into animals it induces the formation of type-specific agglutinins effective against both matt and glossy variants. Such antisera do not, however, give a precipitin reaction with solutions of M substance, nor do they usually protect mice against infection with virulent matt organisms of the same type (4). The only known type-specific immune reaction of antisera prepared with glossy organisms is type-specific agglutination of homologous type matt and glossy strains.

These phenomena have heretofore (4, 6) been explained on the basis of the quantitative relationships involved in the agglutination reaction: *viz.* only a small amount of antibody is required for complete agglutination, and possibly a similarly small amount of agglutinogen is necessary to bring about agglutination; these conditions might account for the type-specific immunological reactions of glossy variants. In the precipitin reaction, on the contrary, a high concentration of antibody is needed for a satisfactory visible precipitate; such a concentration is present in antisera prepared with matt strains but not in those prepared with glossy strains. This explanation did not account satisfactorily for the usual lack of passive protective capacity of antisera prepared with glossy variants, since the small amount of antibody present in highly diluted antisera prepared with matt variants still protects mice effectively. Consequently, by this hypothesis, the trace of M antibody postulated for antisera prepared with glossy strains would be expected to afford considerable passive protection. As a result of a study of type specificity in opsonic reactions, Lyons and Ward suggested that type-specific protection, type-specific precipitin reactions with M, and type-specific reactions due to opsonins are parallel phenomena, but that type-specific agglutination is dependent upon a separate antigen-antibody system (7). Since there was no very exact definition of the nature or mode of action of such an additional system, it has previously seemed to us more satisfactory to base the explanation, as outlined above, on the quantitative relationships of the known M antigen and its antibody (6). Newer im-

munological evidence presented here shows, however, in partial agreement with the suggestion of Lyons and Ward, that a second qualitatively different antigen-antibody system causes type-specific agglutination of both matt and glossy hemolytic streptococci in addition to the type-specific agglutination of the matt variant usually caused by the previously recognized type-specific system, consisting of the M substance and its antibody.

The type-specific antigen of this newly identified system will, for convenience, be designated hereafter as "T" and its antibody as "anti-T," without any implication as to whether T occurs as a separate chemical entity in the matt variant or in conjugation with the M substance. It does, however, occur without the M substance in the glossy variant.

EXPERIMENTAL

Cultures.—The matt and glossy variants were in part those used previously (4) and in part were obtained more recently. The strains used were: type 1, T1 and S118; type 3, T3 and D58 (Richards); type 6, S43; type 14, S23; they were recently described in detail (3 *e*). Experiments, not here recorded, were also performed with numerous other representative strains of the types under consideration.

For simplicity in the tables, strains are indicated as "type" followed by the type number.

The characteristics of the glossy variants were usually the following. Under optimal conditions the colony was glossy; but the requirements for demonstrating the typical glossy colony form seemed subject to so much individual strain variation and to so many slight uncontrollable differences in media that the colony form could only be used as a guide when it happened to show the differential characters. The definition of the variant forms was, therefore, based on the functional differences correlated with these colony forms in previous work. The criteria employed to differentiate glossy variants were as follows: 0.5 cc. of a 12 hour, or younger, broth culture of a glossy variant injected intraperitoneally, killed mice irregularly; type-specific M substance could not be demonstrated in concentrated extracts of the bacteria; antisera prepared by immunizing rabbits with glossy variants did not contain demonstrable M antibody and afforded little or no passive protection to mice infected with virulent matt cultures of the same serological type. These glossy variants either arose spontaneously during cultivation on artificial media, or were developed by serial subculture in broth containing 10 per cent of homologous type-specific immune serum.

The matt variants had the usual properties associated with this form. The cultures were employed either in the form isolated or after their virulence had been enhanced by numerous intraperitoneal passages through white mice. Matt virulent cultures killed mice in doses of 10^{-8} cc. and matt avirulent strains required 10^{-1} or 10^{-2} cc., but both matt variants contained maximal amounts of M substance easily demonstrable in unconcentrated extracts of the bacteria. Both matt virulent and matt avirulent forms had matt or mucoid colonies, depending upon growth conditions, and both were effective antigens in inducing the formation in immunized rabbits of type-specific anti-M precipitins and in bringing about active or passive immunity against infection of mice with virulent homologous type cultures.

Methods

The methods of preparing antisera and the techniques used in the precipitin test were those previously described (8, 9). The preparation of M extracts and the techniques employed in passive protection tests have also been described recently (10); the agglutination technique was essentially the same as that employed formerly; 12 to 16 hour broth cultures, if the growth was sufficiently heavy and diffuse, were used without treatment. If unsatisfactory in either respect, the bacteria were separated from the culture medium by centrifugation and resuspended in fresh broth. A modification of the broth developed by Todd and Hewitt was used for growth and resuspension of these cultures (11). No difficulty was experienced with suspensions of glossy variants, but some of the matt variants, especially the members of type 3, were prone to spontaneous agglutination. One helpful device was to grow these cultures at room temperature overnight and then concentrate them by centrifuging and resuspending the bacteria in the desired volume of fresh broth. Much the same effect was obtained by concentrating the early growth of very young cultures incubated at 37°C. Although no difference was observed in the results of agglutination with cultures prepared in these different ways, a culture grown for 16 hours at 37°C. was used whenever possible. The prepared culture was added in a volume of 0.5 cc. to equal volumes of serum which had been diluted serially in broth. Control series with normal serum, diluted the same as the immune serum, were invariably included, and the experiment was discarded unless this series was negative. Readings were made after incubation for 2 hours at 56°C.

Absorption experiments were performed with either living bacteria or those killed by heating for from 5 minutes to 1 hour at 56°C. Usually two parts of undiluted serum were mixed thoroughly with one part of packed bacteria and incubated at 37°C. for one-half hour. The serum was removed after centrifugation at high speed, and if living bacteria had been used for absorption the serum was sterilized by heating at 56°C. for one-half hour. Comparative tests showed no differences in results attributable to the use of living, as contrasted with heat-killed, bacteria for absorption; but living bacteria were usually employed in the present experiments to avoid possible inactivation of antigens by heat.

Absorption experiments with M solutions were performed as previously described (3 *e*, see Table VI).

Type-Specific Agglutination in Type 1

In order to determine whether any of the type-specific agglutination was dependent on some other antigen than the M substance, a series of absorption experiments was set up using various matt and glossy strains of hemolytic streptococci as absorbing agents.

Absorption Experiment with Whole Bacteria of Homologous and Heterologous Matt and Glossy Variants.—Aliquot parts of serum R45-35, from a rabbit immunized with type 1 matt culture, were mixed with living bacteria centrifuged from 16 hour broth cultures of the strains used (type 1 matt, type 1 glossy, type 3 matt, type 3 glossy, type 6 matt, and type 6 glossy). The mixtures, together with a tube of untreated control serum, were incubated in a water bath at 37°C. for one-half hour, then centrifuged immediately.

The supernatant serum was removed and, in a tightly stoppered tube, completely immersed in a water bath at 56°C. for one-half hour in order to sterilize it. The streptococci were invariably killed by this procedure.

Dilutions of these sera were prepared, and used for agglutinations, precipitin reactions, and passive protection tests in mice.

Results of Type 1 Absorption Experiments.—As shown in Table I, all agglutinins for type 1 matt and for type 1 glossy were removed by absorption with either the type 1 matt or the type 1 glossy variant, but not by absorption with matt or glossy strains of heterologous type. Thus, the type-specific agglutinability and agglutinin-absorbing capacity of type 1 matt and glossy strains were found identical. Fourteen other type 1 matt strains, which yielded type 1 M substance in extracts, did not agglutinate in serum from which the type-specific agglutinin had been absorbed by a type 1 glossy strain, in spite of the fact that this serum still contained anti-M precipitins, as shown by the M precipitin reactions in Table I. In other words, although the type 1 glossy variant removed the type-specific agglutinin just as effectively as did the type 1 matt variant, nevertheless, it did not remove the anti-M precipitin: only absorption with type 1 matt variants removed the anti-M from the serum. From these and numerous other absorption experiments similarly performed, it seems clear that in the case of type 1 the anti-M precipitin does not cause agglutination of type 1 strains, even though these cultures are in the matt phase and contain the M antigen.

It was of interest to determine whether the type 1 type-specific agglutinin, anti-T, which on the basis of the foregoing experiments appeared to be distinct from the anti-M precipitin, had any function in type-specific protection. Evidence on this point was obtained by passive mouse protection experiments, several of which are recorded in Table I. The individual experiment numbers are indicated in order that the separate parts may be compared with the appropriate unabsorbed controls of the same experiment, as well as with similar experiments done at different times. The five different tests made with unabsorbed serum show the amount of variation encountered in different experiments, especially with high dilutions of serum. This is probably due in part to variable excretion or other loss of antibody in the 24 hour interval between the injection of serum and the infection of the mice. Other variables must also be considered, for example, in Experiment I the serum was absorbed twice instead of once as in the other experiments, and in this instance mice not of the Rockefeller Institute stock were used. In this experiment, the whole level of protection was somewhat lower than in the others, but a comparison of the three parts of Experiment I,

TABLE I

Type 1 Matt Antiserum

Absorbed with Homologous and Heterologous Matt and Glossy Strains

Type 1 matt antiserum	*Section A* Agglutinin reaction*												*Section B* Precipitin reaction*		*Section C* Passive protection tests in mice*									
	Strain used in agglutinations												M extract		Strain used to test protection Type 1 matt (10^{-4} cc.)									
	Type 1 matt variant						Type 1 glossy variant								0.5 cc. serum dilution									
	Final serum dilutions																							
	1:40	1:80	1:160	1:320	1:640	1:1280	1:40	1:80	1:160	1:320	1:640	1:1280	Type 1 matt	Type 1 glossy	Experiment No.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Not absorbed	++++	++++	++++	+++	+++	+	++++	++++	++++	++++	+++	++±	++++	−	I†	S	S	S	D8	D1	S	D1	S	D1	D1
															III	S	S	S	S	S	S	S	S	D2	D2
															IV		S	S	S	S	S	S	D3	D12	S
															V	S	S	S	S	S	D4	S	S	D4	D4
															VI	S	S	S	D1	S	S	S	D2	D4	D1
Absorbed with Type 1 matt	−	−	−	−	−	−	−	−	−	−	−	−	−	−	I†	D1	S	D1	D1	D1	D1				
															V		D1	D2	D1	D1	D1	S	D1	S	D1
															VI	D1	D1	D1	D1	D1	D1	D1	D1	D1	D1
" 1 glossy	−	−	−	−	−	−	−	−	−	−	−	−	++++	−	IV		S	S	S	S	S	D2	D5	S	S
															V		S	S	D14	S	S	D2	D1	D1	S
															VI	S	D14	D14	S	S	S	S	D4	D2	D2
" 3 matt	++++	++++	++++	++++	++++	++	++++	++++	++++	+++	+++	+	+++	−	I†	S	S	S	D2	D5	D1	S	D1	D1	D1
" 3 glossy	++++	++++	++++	+++	++	++	++++	++++	++++	++++	+++	+	+++	−	V		S	S	S	S	D4	D4	D4	D2	D2
" 6 matt	++++	++++	++++	+++	++	+	++++	++++	++++	++++	++++	+	+++	−	V		S	S	S	S	S	D4	S	D1	D4
" 6 glossy	++++	++++	++++	+++	++	++	++++	++++	++++	++++	++++	+	+++	−	V		S	S	S	S	S	S	S	S	S

* For details of technique, see "Methods." Blank indicates no test.

In the agglutinin and precipitin reactions, ++++ to + indicate degrees of reaction, − indicates a negative reaction.

M extracts prepared by heating bacteria at 100°C. with N/20 HCl (*cf.* "Methods").

Controls for the agglutinin and precipitin reactions, included in every experiment, were set up with normal serum in the same series of dilutions used in the test and with broth and saline controls. They were all negative.

In the protection tests, S indicates survival for 2 weeks, D with a numeral indicates death within that number of days.

Virulence controls for the protection tests usually included 10 mice each inoculated with 10^{-4} cc. of culture, all of which died regularly overnight, and 2 to 5 mice each with 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} cc. of culture respectively. In these experiments with type 1, the minimal lethal dose was usually 10^{-6} cc. to 10^{-8} cc.

† Test infecting doses of 10^{-2} and 10^{-6} cc. yielded similar results in Experiment I. This experiment was somewhat different in technique from the others in that 2 successive absorptions were made and a strain of mice different from the Rockefeller Institute strain was used. These differences may explain the lower level of protection in this experiment.

together with the other protection experiments recorded in Table I, shows the following results: Serum absorbed with homologous type 1 matt organisms lost its protective property for type 1, but serum absorbed with type 1 glossy bacteria protected just as well as unabsorbed serum although the type-specific agglutinins were removed in both instances. Other control absorptions with heterologous matt and glossy strains, types 3 and 6, failed to remove either the protective antibodies or the agglutinins. The parallelism between the presence of M antibody and protective power, and the lack of correlation between these properties and the type-specific agglutinin, anti-T, suggest that anti-M is the protective antibody and, further, that in the case of type 1, anti-T is not essential to protection, since its removal by absorption with type 1 glossy variants does not destroy the protective capacity of the serum.

Absorption Experiment with M Solutions

In analyzing the relationship of these two antigenic systems, type 1 serum was absorbed with the type-specific protein M in solution to determine whether the protective antibodies and the agglutinins might be removed simultaneously. Previous work (3 *e*) had shown that protective antibody was removed by such an absorption.

Type 1 matt antiserum was absorbed with M solution by the method of optimal proportions (12). In a preliminary test a 1:10 dilution of serum was mixed with varying amounts of a 1:10 dilution of M extract and the first tube showing flocculation was noted. Whole serum was then absorbed by adding a concentrated solution containing M in a proportion double that required for the fastest flocculation observed in the preliminary test. The mixture was incubated at 37°C. for 1 hour and refrigerated overnight. After centrifugation, the supernatant fluid was removed and tested for agglutinin, precipitin, and protective antibody. Three such experiments were performed, two with the same lot of type 1 serum and the third with another prepared against a different type 1 strain. Serum R45-86, from a rabbit immunized with a type 1 strain, S118, was absorbed with M extract of the same strain and used in a typical experiment (Table II).

Section A of this table shows that the unabsorbed serum gave typical type-specific precipitin reactions with M solution, whereas serum absorbed with M failed to react with M antigen; section B correspondingly shows that the removal of the type-specific anti-M precipitin also removed the protective antibody. In contrast, section C demonstrates that these same lots of absorbed and unabsorbed sera were equally active in agglutinating the homologous type 1 matt and glossy organisms. This shows that the two type-specific systems can be separated and are independent of each other.

TABLE II

Type 1 Matt Antiserum

Absorbed with Homologous M Solution

A. Precipitin reactions

Type 1 matt antiserum 0.1 cc.	Type 1 M extract, 0.1 cc. diluted									
	1:1	1:2.5	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Not absorbed	++++	++++	+++	++	+	±	−	−	−	−
Absorbed with type 1 M solution	−	−	−	−	−	−	−	−	−	−

B. Passive protection tests in mice

Infecting culture	Type 1 matt antiserum	0.5 cc. serum diluted									
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Type 1 matt (strain S118) 10^{-4} cc.	Not absorbed	S	S	S	S	S	S	D1	D1	D2	D1
	Absorbed with type 1 M solution	D2	D3	D1	D3	S	D1	D1	D1	D1	D1

C. Agglutinin reactions

Culture used in agglutination	Type 1 matt antiserum	Final serum dilutions							
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Type 1 matt*	Not absorbed	++++	++++	++++	++++	++++	++	+	±
	Absorbed with type 1 M solution	++++	++++	++++	++++	+++	++	+	−

See Table I for footnotes.

* Same result if type 1 glossy is used as the test organism in the agglutinations.

TABLE III

Type 1 Glossy Antiserum

Absorbed with Matt and Glossy Strains

Type 1 glossy antiserum	Agglutinin reactions: Strains used in agglutinations														Precipitin reactions with type-specific M substance from type 1 matt variant
	Type 1 matt variant							Type 1 glossy variant							
	Final serum dilutions														
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	
Not absorbed	++++	++++	++++	++++	++++	+++	++	++++	++++	++++	++++	++++	+++	++	−
Absorbed with strain															
Type 1 matt	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 1 glossy	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
" 3 matt	++++	++++	++++	+++	+++	++	++	++++	++++	++++	+++	+++	++	+	−
" 6 matt	++++	++++	++++	++++	++++	+++	++	++++	++++	++++	++++	++++	+++	++	−

See Table I for footnotes.

From these results, it was obvious that an agglutinogen must be present in the glossy variant, as well as in the matt. The serological reactions of rabbits immunized with a type 1 glossy strain bore out this hypothesis.

Immunization of Rabbits with Type 1 Glossy Variant

Four rabbits, in two different experiments, were immunized with whole bacteria of a type 1 glossy strain in extracts of which no type-specific M substance could be detected. All the rabbits developed agglutinins of high titer which were not absorbed by heterologous type bacteria, but were absorbed equally well by either matt or glossy variants of homologous type 1 strains (Table III). In accord with previous work on the antigenicity of glossy strains (4), these anti-glossy-variant sera contained no demonstrable anti-M precipitin, but some of them, in doses of 0.5 cc., protected part of the mice tested with 10 to 100 M.L.D. of the homologous type 1 matt organisms. The specificity of this protection and the antibody responsible for it have not been determined.

Type-Specific Agglutination in Three Other Specific Types

It was desirable to determine whether the failure of M antibody to cause agglutination of type 1 strains is a general phenomenon or is characteristic only of type 1. Consequently, types 3, 6, and 14 were used in absorption and protection experiments similar to those described for type 1.

Absorption Experiment with Types 3, 6, and 14.—Comparable absorption experiments were made with immune sera R46-71 and R46-49, from rabbits immunized respectively with two different matt virulent type 3 strains (T3 and P279), and with serum R45-74 from a rabbit immunized with a matt virulent type 14 strain (S23). The general plan was to absorb one portion of serum with M solution of homologous type and another portion with bacteria of the homologous glossy variant. Other portions were absorbed with heterologous matt and glossy strains (type 1 or type 6); and, in addition, aliquot parts of serum already absorbed with M solution of type 14 were further absorbed with whole bacteria from the glossy variants of types 6 and 14 respectively. Type 6 serum, R44-57, prepared with the matt variant of strain S43, was similarly absorbed with bacteria of matt and glossy variants.

The technique used was similar to that employed in the experiments with type 1, and included suitable controls absorbed with the homologous matt strains and controls of unabsorbed serum.

The agglutinating and precipitating capacities of these absorbed sera are shown in Table IV. The precipitin reactions, recorded in column 5, agree with previous similar experiments: homologous M solutions and homologous matt organisms absorbed the type-specific M antibody in every case (tubes B and C, types 3, 6, and 14 sera). Absorption with homologous

TABLE IV

Type-Specific Precipitins and Agglutinins in Absorbed and Unabsorbed Sera, Types 3, 6, and 14

Type serum	Tube identification	Serum anti-matt variant — Treatment of serum	Tested with strain or extract of type	Precipitin reactions with type-specific M substance from homologous matt variant	Agglutinin reactions — Strain used in agglutinations — Homologous matt variant — Final serum dilutions 1:40	1:80	1:160	1:320	1:640	1:1280	Homologous glossy variant — Final serum dilutions 1:40	1:80	1:160	1:320	1:640	1:1280
3	A	Not absorbed	3	+++	++++	++++	++++	++++	+++	++	++++	++++	++++	++++	+++	++
		Absorbed with															
	B	M solution from type 3 matt		−	++++	+++	+++	++	+	±	++++	++++	++++	++	+	−
	C	Bacteria: type 3 matt		−	−	−	−	−	−	−	−	−	−	−	−	−
	D	" type 3 glossy		++	+++	+++	+++	+++	++	+	−	−	−	−	−	−
	E	" type 1 matt		++	++++	++++	++++	++++	+++	++	++++	++++	++++	++++	+++	++
14	A	Not absorbed	14	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+
		Absorbed with															
	B	M solution from type 14 matt		−	++++	++++	++++	+++	++	+	++++	++++	++++	+++	++	±
	C	Bacteria: type 14 matt		−	−	−	−	−	−	−	−	−	−	−	−	−
	D	" type 14 glossy		++	+++	+++	++++	+++	+	+	−	−	−	−	−	−
	E	" type 6 matt		++	++++	++++	++++	+++	+++	+++	++++	++++	++++	+++	++	+
	F	" type 6 glossy		++	++	++	++	++	++	+	+++	++++	+++	++	+	±
	BD	M solution from type 14 matt, then bacteria, type 14 glossy		−	+++	++*	−	−	−	−	−	−	−	−	−	−
	BF	M solution from type 14 matt, then bacteria, type 6 glossy		−	++++	++++	+++	++	±	−	+++	++	++	+	±	−
6	A	Not absorbed	6	+++	++++	++++	++++	++++	++++	++	++++	++++	++++	++++	++++	++++
		Absorbed with															
	C	Bacteria: type 6 matt		−	−	−	−	−	−	−	−	−	−	−	−	−
	D	" type 6 glossy		++	+++	++	++	±	−	−	−	−	−	−	−	−
	E	" type 1 matt		++	++++	++++	++++	++++	++++	++	++++	++++	++++	++++	++++	++++

See Table I for footnotes.

See Table V for protection tests with some of these sera.

* Probably incomplete absorption with M solution.

glossy bacteria (tubes D), as well as with heterologous matt and glossy bacteria (tubes E and F), failed to affect this reaction significantly. These findings correspond with those obtained with type 1.

The agglutination reactions of types 3, 6, and 14, however, were different from those of type 1. Among these types the M antibody, as well as the T antibody, agglutinated matt cultures. This is demonstrated by the fact that sera absorbed with the homologous glossy strain (tube D in each of these experiments) no longer agglutinated the homologous glossy, because the anti-T had been removed, but still agglutinated the homologous matt to a somewhat lower titer on account of the anti-M remaining in the serum. Removal of both anti-M and anti-T by absorption with the homologous matt variant resulted in loss of all type-specific agglutinins for both of the homologous type variants (tube C).

In the experiment with type 14, a combination absorption, first with homologous M solution and second with homologous glossy bacteria (tube BD), removed almost all agglutinins for homologous matt strains. The control lot (tube BF), in which the absorption with M solution was followed by absorption with bacteria from a heterologous glossy strain, still agglutinated both matt and glossy variants of type 14, because the type-specific anti-T agglutinin was still present.

Taken altogether, the experiments recorded in Table IV indicate with considerable certainty that, in contrast to the finding with type 1, the M antibodies in types 3, 6, and 14 cause part of the type-specific agglutination of matt strains in anti-matt sera but do not bring about the agglutination of glossy variants. The latter are agglutinated by a separate kind of antibody, anti-T, which they absorb specifically from homologous type sera; and matt variants of the same type are also subject to agglutination by this antibody and absorb it from the serum. Although pertinent experiments are not detailed here except with type 1, observations have been made on known glossy strains in past years to indicate that anti-glossy-variant sera of these and other types contain the anti-T agglutinin elicited by the T antigen common to matt and glossy variants of the same type, but these sera do not contain the M antibody characteristic of anti-matt-variant sera.

Numerous passive protection tests in mice were performed with types 3 and 14 sera variously absorbed. Table V, column 3, compared with Table IV, column 2, shows the correspondence between the protection tests and the precipitin and agglutinin reactions of type 14. Serum lots B, BD, and BF, absorbed with the homologous matt strain or M solution, no longer protected mice against infection with 10,000 M.L.D. of the homologous type 14 matt strain; although unabsorbed serum (lot A), in a dilution of 1:32,

and serum absorbed with homologous or with heterologous glossy variants (lots D and F) protected mice approximately equally well against such infection. Thus the protective capacity of the serum paralleled the presence of M antibody but appeared unrelated to the presence of the T antibody.

TABLE V

Passive Protection Tests in Mice with Type 14 Antiserum Absorbed in Various Ways

Infecting culture		Tube identification (*cf.* Table IV)	Type 14 matt antiserum	0.5 cc. serum dilution									
Strain	Dose			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
	cc.												
Type 14 matt	10^{-4}	Type 14, A	Not absorbed		S	D2	S	S	D13	D8	D13	D1	D1
	"	" B	Absorbed with M solution from type 14 matt	D8	D1	S	D1	D2	D2	D1	D2	D1	D1
	"	" D	Bacteria: type 14 glossy	S	D5	S	S	S	S	D2	D4	D5	D1
	"	" F	" type 6 glossy	S	S	S	S	D1	D1	D2	S	D1	S
	"	" BD	M solution from type 14 matt, then bacteria type 14 glossy			D5	D1	D1	D1	D2	D1	D1	D1
	"	" BF	M solution from type 14 matt, then bacteria type 6 glossy			D3	D1	D1	D1	D1	D1	D1	D1

				Mouse No.							
				1	2	3	4	5	6	7	8
	10^{-4}		Virulence controls, no serum	D1	D1	D1	D1	D1	D1	D1	D1
	10^{-6}		" "	D1	D1	D4					
	10^{-7}		" "	D1	D3	S					
	10^{-8}		" "	D1	D3	S					

Protection tests with these same lots of absorbed serum repeated on another day gave essentially the same results.

See Table I for footnotes.

See Table IV for agglutinin and precipitin reactions.

Similar protection experiments were done with the type 3 serum, the precipitin and agglutinin reactions of which are recorded in Table IV, and with other type 3 sera. The results were not as consistent as those recorded for type 14, probably on account of technical difficulties, but they pointed in the same direction. Experiments of this nature have not yet been made with other types.

DISCUSSION

The present additional analysis of the immunological reactions of the matt variants of group A hemolytic streptococci and their degraded glossy

derivatives indicates the existence of two qualitatively different type-specific antigenic systems, both of which are present in the matt variants but only one in the glossy. In most types so far studied, antibody to the type-specific protein M, found in matt variants, and antibody to the newly recognized part of the antigenic complex designated as T and present in both matt and glossy variants, may cause type-specific agglutination of homologous matt organisms, but only T antibody brings about type-specific agglutination of the glossy form. In type 1, however, this latter (T) antibody is the only one which causes type-specific agglutination, although the M substance can be demonstrated in type 1 matt cultures used for agglutinations and the corresponding antibody in antisera prepared with such cultures. Furthermore, in the case of type 1, a combination of M antigen and antibody occurs, as shown by absorption experiments, but the second, or flocculation, phase of the reaction does not follow; the reason for this is unknown. Because of this peculiarity, however, matt and glossy variants of type 1 strains show no difference in type-specific agglutination. In other types, on the contrary, serum from which the T antibody has been absorbed by the glossy variant still agglutinates the matt variant on account of the M anti-M system, but does not agglutinate the glossy variant. In spite of this difference in agglutination between matt and glossy variants of most types studied, the glossy derivative retains the type specificity of the parent organism from which it was derived, possibly because the remaining type-specific antigen T, in the degraded glossy variant, may constitute part of a more complex substance in the matt variant, composed partly of M and T in combination. There is, at present, no evidence to indicate whether this possibility is correct or whether the two type-specific factors found in the matt variant occur as individual chemical entities. In either case, however, separate antibodies to the two components, M and T, are formed. A summary of these facts is given in Table VI, for type 1, and in Table VII for the other types so far subjected to this kind of study.

In the past, most investigators have considered that one type-specific antigen-antibody system was responsible for all of the type-specific reactions of group A hemolytic streptococci. Several years ago, however, Lyons and Ward suggested that type-specific agglutination was dependent upon an entirely different antibody from the one responsible for the M precipitin reaction, for protection, and for type-specific opsonic reactions. The present conception of two type-specific antigen-antibody systems differs somewhat from their idea, in that M antibody, in all types studied except type 1, causes part of the type-specific agglutination, although the newly identified T antibody seems to be chiefly responsible for this reaction.

TABLE VI

Summary of Type-Specific Reactions of Group A, Type 1, Hemolytic Streptococcus Absorption Experiment

Type 1 serum	Agglutinations with		M precipitin reactions with		Passive protection in mice against infection with
	Type 1 matt culture	Type 1 glossy culture	Type 1 matt M extract	Type 1 glossy M extract	Type 1 matt culture
Type 1 Matt Antiserum					
Control: not absorbed	++++	++++	++++	−	Survived
Absorbed with bacteria					
Homologous strain: type 1 matt	−	−	−	−	Died
Homologous strain: type 1 glossy	−	−	++++	−	Survived
Heterologous strain: type 6 matt	++++	++++	++++	−	Survived
Heterologous strain: type 6 glossy	++++	++++	++++	−	Survived
Type 1 Glossy Antiserum					
Control: not absorbed	++++	++++	−	−	Usually died
Absorbed with bacteria					
Homologous strain: type 1 matt	−	−	−	−	
Homologous strain: type 1 glossy	−	−	−	−	
Heterologous strain: type 6 matt	++++	++++	−	−	
Heterologous strain: type 6 glossy	++++	++++	−	−	

TABLE VII

Summary of Type-Specific Reactions Typical of Other Types Studied: Types 3, 6, 14 Absorption Experiment with Type 6 Matt Antiserum

Type 6 serum	Agglutinations with		M precipitin reactions with		Passive protection tests in mice against infection with
	Type 6 matt culture	Type 6 glossy culture	Type 6 matt M extract	Type 6 glossy M extract	Type 6 matt culture
Control: not absorbed	++++	++++	++++	−	Survived
Absorbed with bacteria					
Homologous strain: type 6 matt	−	−	−	−	Died
Homologous strain: type 6 glossy	++	−	++++	−	Survived
Heterologous strain: type 1 matt	++++	++++	++++	−	"
Heterologous strain: type 1 glossy	++++	++++	++++	−	"

If the strains that Lyons and Ward studied were members of type 1, or of some other similar type in which anti-M does not cause agglutination, the two type-specific antigen-antibody systems postulated by them were

probably analogous to those reported here. Since the types they used were not identified, it is not possible to analyze their data in terms of the T antigen. The question of whether T antibody functions as an opsonin has not been investigated.

While previous work has shown that the M substance is a protein, capable of giving good type-specific precipitin reactions, the type-specific antigen T has not been identified chemically or even obtained in a form suitable for precipitin reactions. In the bacterial cell, it appears to be one of the most active antigens present, as shown by the ease with which agglutinating antibodies are produced in rabbits in contrast to the usually more tedious and arduous task of producing M antibody.

Recognition of this second factor in type-specific agglutination has solved the long standing puzzle as to why glossy variants agglutinate type specifically and, on injection into rabbits, give rise to type-specific agglutinins effective against both matt and glossy forms of the organism. It would appear that the glossy variant may be the desirable form to use in preparing type-specific agglutinating antisera, but the difficulty of obtaining this variant at will from all strains is at present an obstacle. If this difficulty could be overcome, the fact that glossy variants almost invariably form diffuse suspensions, which usually agglutinate more rapidly and more completely than the corresponding matt organisms, would offer another approach to facilitating the type differentiation of group A hemolytic streptococci. The objection to this use of glossy variants is that immunological reactions with them would not give a complete picture of the type-specific antigenic complex of these organisms and might occasionally be complicated by the presence of extra antigens, as will be illustrated in the succeeding paper in the case of strain C203. It is also true that glossy variants are more readily subject to the influence of the several non-type-specific agglutinins, which may be present in the antisera, than are matt variants; and this objection would have to be met by proper selection or absorption of type-specific antisera to eliminate non-type-specific antibody.

This analysis of the type-specific immunological reactions of matt and glossy variants still fails to account for the inability of certain occasional strains, especially when freshly isolated from disease, to agglutinate at all, even though the type can be determined by other immunological reactions, such as M precipitin reaction, protection tests, and antigenicity. Although it is possible in certain cases that this may be due to an inhibitory action of the mucoid polysaccharide, it is hard to explain why most strains containing this substance are, nevertheless, agglutinable. Search for additional unrecognized chemical constituents of the cell affecting its agglu-

tinability has been stimulated by the fruitful investigations in recent years of the Vi antigens of the Salmonellas, and it is obvious that further work in this direction should be undertaken with streptococci.

Study of the two type-specific parts so far recognized in the antigenic complex of group A hemolytic streptococci has shown that antibody to the type-specific protein, M, found in matt variants only, is responsible for type-specific protection. The second part of the type-specific complex, the T agglutinogen, is found in matt variants together with the M antigen. It is the only part of the type-specific complex identified in glossy variants, and in neither case is it apparently involved in protection. Since the T antigen seems to be the chief, and in type 1 the only, factor in type-specific agglutination, it is obviously futile to try to estimate the protective capacity of streptococcus antiserum by its agglutinin titer. Any adequate measure of this property must be based upon an assay of the M antibody.

SUMMARY

1. Two qualitatively different type-specific antigens, designated M and T, have been found present in matt variants of group A hemolytic streptococci, but only one of these, the T antigen, occurs in the degraded glossy variant.

2. The protein nature of the M antigen, present in matt variants only, has been demonstrated in previous work, but the chemical characteristics of the newly recognized antigenic factor, T, present in both variants, have not been determined. This T factor is identified only by its immunological reactions. It is unknown whether the two type-specific antigenic factors, M and T, occur as separate chemical entities in the matt variant or in conjugation.

3. Antibody to the type-specific protein, M, appears responsible for the M precipitin reaction, for type-specific protection, and, as a rule, for part of the type-specific agglutination of matt variants, but in type 1 it does not cause agglutination.

4. Antibody to the second type-specific antigen, T, seems to be solely responsible for type-specific agglutination of the glossy form and to play a large rôle in type-specific agglutination of the matt form, but apparently it is not involved in protection. This T antibody causes all of the type-specific agglutination of type 1. Consequently, type 1 matt and glossy variants agglutinate and absorb agglutinin alike, and antisera to both are identical in content of type-specific agglutinin though they differ in respect to M antibody.

5. Recognition of the principle underlying type-specific agglutination of glossy variants makes it possible to suggest, with certain reservations, the

use of glossy variants for type classification by agglutination. These variants are suitable for preparing type-specific agglutinating antisera, and they form stable suspensions for use in the reaction. Improved methods are needed for deriving glossy from matt variants.

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