THE OCCURRENCE OF TWO M ANTIGENS IN CERTAIN GROUP A STREPTOCOCCI RELATED TO TYPE 14

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The type classification of Group A streptococci depends upon the M proteins, which are immunologically different for each type. These antigens are of special importance among the several well studied antigens of this group of streptococci because of their close association with virulence. They are demonstrable by precipitin tests, and antibodies to them react type-specifically in active and passive animal protection tests and in ingestion and bactericidal tests. There have been described, up to the present time, some 45 types of streptococci, and in each type the determinant has been a single M antigen characteristic of the type.

The work reported here shows that most Type 14 streptococci possess two M antigens, the presently recognized M antigen of Type 14 and another M designated here Type 51. A few strains have been encountered possessing the Type 51 M antigen without the Type 14 M antigen and one strain possessed the Type 14 M antigen alone. Although it has previously been shown that Group A streptococci may possess more than one T antigen (1), the present work is the first demonstration that strains may have two distinct M antigens.

Materials and Methods

Streptococcal Strains.—The principle strains used in this work are listed in Table I. In addition to these, 34 strains obtained from many sources and covering a wide geographical distribution were tested for their M antigen composition. All strains were preserved by freezing and drying. For routine work strains were grown in neopeptone–sheep blood broth and maintained in the refrigerator at approximately 4°C. To maintain cultures capable of vigorous multiplication in normal human blood, strains were recovered approximately once a month from the dried state, or as an alternative method, stock blood broth cultures were sometimes given a single intraperitoneal mouse passage.

Precipitin tests were performed by the capillary precipitin method using crude acid extracts (2) or M extracts (3).

Antisera were prepared by injecting rabbits three times weekly with heat-killed bacterial suspensions, and collecting serum when adequate antibody content had been achieved. Sera were absorbed with packed heat-killed cells in the desired proportions. We are indebted to the Communicable Disease Center, Chamblee, Georgia for absorbed typing sera for many streptococcal types and to Dr. Rebecca C. Lancefield for a generous supply of antiserum prepared against strain Type 14/46.

Bactericidal Tests.—Two types of bactericidal tests have been employed in this study. The

first, modified from Todd (4), demonstrates the ability or inability of strains to grow in human blood in the absence of specific antibody and is, in a sense, a test of the virulence of the strain. It depends on the ability of the strain to resist ingestion and destruction by human phagocytes of peripheral blood.

In the other test, anti-M antibody is included in the reaction mixture, and the ability of this anti-M antibody to foster phagocytosis and consequent destruction of cocci that are otherwise resistant to phagocytosis, is measured. When the test strain is known to possess M

TABLE I

Proposed Antigenic Composition of Type 14 and Related Strains

Strain	M antigens present	Type	Source
AD242/28/2	14, Col. 7	14–51	Derived by 28 mouse passages from a strain isolated by Dr. Charles H. Rammelkamp, Jr., from an acute upper respiratory infection, Warren Air Base, Wyoming, 1951
AD242/28/8	14, Col. 7	14–51	
Colony 3	14, Col. 7	14–51	Obtained by picking colonies from a blood agar streak of strain AD242/28/8
Colony 7	Col. 7	51	
AD309	Col. 7	51	Bainbridge Naval Training Center, 1952. Isolated by Dr. Horace Gezon
Type 14	14, Col. 7	14–51	Dr. Rebecca C. Lancefield
Type 14/47	14, Col. 7	14–51	
Colindale 8199 (Strain Lowe)	14	14	National Collection of Type Cultures, Streptococcal Reference Laboratory of the Public Health Laboratory Service, London, England

The colony 7 M antigen and the Type 51 M antigen are identical.

The figure following the first diagonal indicates the number of intraperitoneal mouse passages. The figure following the second diagonal indicates the number of subcultures in blood broth.

antigen, the test is a measure of anti-M antibodies in the serum. When a serum is known to possess anti-M antibodies the test indicates the presence of that M antigen on the test strain. When both the M antigen of a strain and the antibody content of the serum are unknown quantities, a positive result in the test (inhibition of growth) is indicative of the operation of an M–anti-M system (5, 6). In this test a modification of the indirect bactericidal test of Rothbard (7) was used employing normal human blood and rabbit antisera. Cultures were prepared by inoculating nine volumes of filtered beef heart infusion broth (8) containing 20 per cent normal rabbit serum with one volume of overnight blood broth culture, incubating for 2 hours at 37°C. and resuspending the cocci in fresh filtered broth. From this suspension, 10-fold serial dilutions were made. In some tests dilutions from 10^{-2} to 10^{-7} ml., inclusive, served as inoculum sources. In others, where smaller and more closely spaced inocula were desired, 2-fold dilutions were prepared, either from the 10^{-4} or the 10^{-5} ml. dilutions, depending upon the density of the resuspended culture. Suspensions were kept immersed in ice water

until ready for use. The number of chains inoculated was determined from colony counts in blood agar. 0.1, 0.05, and 0.25 ml. of coccal suspension, serum, and heparinized normal human blood (heparin in final concentration of 1:11,000), respectively, were rotated at 8 R.P.M. in 7 × 70 mm. plain glass tubes, closed with silicone stoppers, at 37°C. for 3 hours. Then, 0.1 ml. from each tube was plated in blood agar. After incubating the plates overnight, the number of colonies was counted and the result multiplied by 4 to obtain the total number of chains present in the 0.4 ml. of rotated mixture. When too many colonies were present to count, the plates were read as laked, partially laked, or too many to count accurately. Such results appear in the tables as L, PL, and TM, respectively.

Ingestion Tests.—The details of this test, which have been described in a previous paper (8), were essentially the same in this work, except that whole blood was substituted for plasma-leucocyte suspension to make the test correspond more closely to the bactericidal test.

Long-Chain Tests.—The long-chain test devised by Stollerman (9) depends on the fact that streptococci grown in the presence of homologous anti-M serum form longer chains than are formed when normal serum is used. The test is performed by counting the average number of cocci per chain in streptococcus-serum mixtures grown in test tubes. A corresponding phenomenon is observed in glass slide preparations of the type previously described from this laboratory (10), but in this case individual chains of the inoculated streptococci grow out as separate microcolonies. If a streptococcal strain grows in microcolonies composed of short chains in normal serum, the microcolonies are generally composed of long chains when homologous anti-M serum is substituted for normal serum, or serum of heterologous type.

EXPERIMENTAL

A strain of Type 14 streptococcus which had received 28 passages through mice (AD242/28/2) was observed to grow largely in long chains in the presence of homologous rabbit antiserum in glass slide preparations. The strain was maintained as a stock culture in blood broth at 4°C., and monthly transfers were made in blood broth. After 6 such transfers the strain (AD242/28/8) when grown in the presence of the same serum, was observed to form short chain microcolonies as well as long chain ones. Since the long chain phenomenon is thought to be a function of the M–anti-M system (11) the possibility of variation, with loss of Type 14 M antigen from some cocci, seemed a reasonable explanation for the increased numbers of short chain microcolonies. Thus, the simultaneous occurrence of long and short chains could be explained by a mixture of Type 14 M^+ and M^- cocci in the culture.

The presumably mixed stock culture, AD242/28/8, was streaked on Todd-Hewitt sheep blood agar to see whether two types of colony were present, but all the colonies were mucoid or postmucoid, and there was no reason to suspect by colony form alone that the culture was mixed.

Twelve well isolated colonies were picked to blood broth (labelled Colony 1 to Colony 12) and the resulting cultures were studied in several ways.

Precipitin and Long Chain Tests.—Crude acid extracts were made from the twelve subcultures of AD242/28/8 and were tested by precipitin reactions with the Type 14 absorbed antiserum used for routine typing. Seven of these gave strong reactions, two reacted weakly

and three failed to react. When the same cultures were grown in the presence of antiserum prepared against Type 14 antiserum, 9 grew as a mixture of long chain microcolonies with occasional short chain ones and 3 grew only in the short chain form. The three latter cultures were the same that failed to give a precipitin reaction with the Type 14 anti-M serum.

TABLE II

Summary of Tests with AD242/28 Cultures

Culture	Precipitin tests Type 14 serum	Mouse virulence LD_{50}	Ingestion tests* per cent neutrophils with cocci		Bactericidal tests					
			15 min.	30 min.						
AD242/28/2 (Maintained in the dried state)	+++	< 11 chains	1	19	Inoculum No. of chains after 3 hrs.	140,000 L	14,000 L	1,400 L	140 L	14 PL
AD242/28/8 (Maintained in blood broth for 6 mos. at 4°C.)	+++	< 9 chains	40	97	Inoculum No. of chains after 3 hrs.	200,000 L	20,000 L	2,000 L	200 L	20 TM
Colonies from the AD242/28/8 culture										
Colony 3	+++	< 7 chains	0	1	Inoculum No. of chains after 3 hrs.	170,000 L	17,000 L	1,700 L	170 L	17 TM
Colony 7	–	590 chains	18	99	Inoculum No. of chains after 3 hrs.	180,000 L	18,000 L	1,800 L	180 768	18 460
Colony 7/16	–	897 chains	2	30	Inoculum No. of chains after 3 hrs.	112,000 L	11,200 L	1,120 L	116 TM	16 1252
Colony 8	–	ND	11	94	Inoculum No. of chains after 3 hrs.	130,000 L	13,000 L	1,300 PL	130 908	13 132
Colony 9	+++	ND	1	0	Inoculum No. of chains after 3 hrs.	180,000 L	18,000 L	1,800 L	180 L	18 PL
327W‡	Gr. A Type 1, agg. only	> 25 million chains	99	100	Inoculum No. of chains after 3 hrs.	140,000 100	14,000 2	1,400 0	140 0	14 0

* Inoculum: 7 million chains.

‡ 327W: Cocci of this strain are highly susceptible to ingestion and the bactercidal effects of normal human blood. The data are included in the table for purposes of comparison.

ND = not done.

Extracts were made from subcultures of 24 well isolated colonies of the initial cultures, AD242/28/2, which had been preserved in the dried state. All the extracts reacted strongly with the Type 14 anti-M serum.

It was assumed, therefore, that the original mouse passage culture on subsequent storage at 4°C., and serial transfer in blood broth, had undergone variation and become a mixture of Type 14 M^+ and M^- variants. For further study an M^+ variant (colony 3) and a presumed M^- variant (colony 7) were selected as representative, but some studies were done with others of the subcultures as well.

Mouse Virulence Tests.—The initial culture, AD242/28/2, was highly virulent for mice when injected intraperitoneally, the LD_{50} being less than 11 chains. The stored stock culture AD242/28/8, which we believed to be a mixture of M^+ and M^- variants, was also highly virulent, having an LD_{50} of less than 9 chains, and we presumed this retention of virulence to be due to the presence of the M^+ cocci. The M^+ variant colony 3 was as highly virulent as the initial culture. Because it is well established that loss of the ability to synthesize M antigen results in a great loss of virulence we expected that the presumed M^- variant, colony 7, would have little power to kill mice. Its LD_{50}, however, was 590 chains, which constitutes a rather high degree of virulence, although not maximal for Group A streptococci.

Ingestion and Bactericidal Tests.—The ability of streptococci to resist phagocytosis and subsequent destruction, as shown in ingestion and bactericidal tests, depends, in large part at least, on the presence of M antigen in the test strains. These tests were therefore performed on the cultures in question with results summarized in Table II. The initial strain AD242/28/2 was highly resistant to phagocytosis in the ingestion test and grew well in human blood in the bactericidal test. The mixed culture AD242/28/8 also grew well in human blood, but considerable phagocytosis was observed in the ingestion test. The latter result is that observed with strains of moderate virulence. The M^+ variants, colonies 3 and 9, reacted in bactericidal and ingestion tests like the initial strain, whereas the presumably M^- variants, colonies 7 and 8, grew somewhat less well in the bactericidal test and after 30 minutes' rotation in the ingestion test showed extensive phagocytosis. Chiefly on the basis of results of the ingestion test, it appeared that the culture AD242/28/8 was, indeed, different from the initial culture, and that it consisted of a mixture of highly virulent cocci possessing abundant M antigen together with moderately virulent cocci in which M was not demonstrated and which were rather readily phagocytized (although not as readily phagocytized as the Type 1 M^- strain 327W).

The possibility existed that the presumably M^- variant, colony 7, was an external contaminant belonging to another serological type, but tests of an M extract of it against sera of all the available types were negative. Sera were not available for Types 8, 9, 11, 25, 27, 34, 35, 37, 40, 42, 44, or 49, but strains known to produce the M antigens of these types were at hand, and extracts of these were tested subsequently with antiserum prepared with the colony 7 strain. Minor reactions occurred with Type 2 and Type 46 M extracts, but these were considered non-specific. It was concluded that colony 7 did not belong to any currently known type.

The colony 7 variant was given 17 intraperitoneal passages through mice and was again tested for its susceptibility to phagocytosis by human leukocytes. In the ingestion test, phagocytosis was accomplished by 2 and 30 per cent of the neutrophils after 15 and 30 minutes rotation, respectively. This represents a considerable increase in resistance to ingestion, but is not as great as that shown by colony 3 cocci (Table II). The mouse passages of colony 7 did not result in the renewed ability of the strain to synthesize detectable amounts of the M antigen of colony 3.

Antibodies Produced by Colony 3 and Colony 7 Strains.—The possibility remained that small amounts of Type 14 M antigen, too small to be detected in a precipitin test, were present on the colony 7 cocci, and could account for their mouse virulence and their ability to grow in human blood. Therefore antisera were prepared by immunizing rabbits with a vaccine of colony 7, and for purposes of comparison, with colony 3.

As shown in Table III, unabsorbed sera for colonies 3 and 7 cross-reacted in precipitin tests. The antisera against colony 7 were absorbed with a Group A strain of heterologous type to remove anti-C and non-specific antibodies and were tested with M extracts of colony 7 and colony 3 organisms. Good reactions were obtained with both extracts. This suggested that colonies 3 and 7 shared a specific precipitating antigen in common, and it was to be expected that anti-

TABLE III

Homologous and Reciprocal Absorptions of Colony 3 and Colony 7 Antisera

Precipitin tests

Antisera	M extracts	
	Colony 3	Colony 7
Colony 3		
Unabsorbed	++++	+++
Absorbed with S43	+++±	++
Absorbed with colony 3, once (1:5)	+	+
twice (1:5)	−	−
Absorbed with colony 7, once (1:3)	+++±	−
twice (1:3)	+++±	−
three times (1:3)	+++	−
Colony 7		
Unabsorbed	++++	+++
Absorbed with S43	+++	++±
Absorbed with colony 3, once (1:5)	++	++
twice (1:5)	−	−
Absorbed with colony 7, once (1:5)	+	+
twice (1:5)	−	−

Overnight readings are given on a scale of − to 4 plus.

serum prepared with colony 3 vaccines would possess the same antibody. However, most bleedings of the colony 3 rabbits after absorption with the heterologous strains gave feeble or no reactions with colony 7 M extract. One rabbit out of 11 immunized with colony 3 cocci produced colony 7 antibodies in moderate concentration, and bleedings from this rabbit were used in subsequent studies.

Absorption of colony 3 antiserum with colony 7 cocci removed precipitating antibodies for colony 7 extracts, but the serum still reacted with extracts of

colony 3 cocci. Absorption of colony 7 antiserum with colony 3 cocci removed precipitating antibodies for extracts of both colony 3 and colony 7 cocci. Homologous absorptions removed all the precipitins in each case. These results suggested that the colony 3 cocci carried two precipitating antigens, one being present in both colony 3 and colony 7 cocci, the other being present only in colony 3 and not in colony 7 cocci. Colony 7 therefore had but a single antigen of this kind, which also occurred in colony 3 cocci.

Bactericidal Tests.—The bactericidal test has been shown to be a satisfactory method of demonstrating the possession of M antigen by a streptococcal strain and can be substituted for the more cumbersome mouse protection test (5, 6).

TABLE IV

Tests of Antisera Prepared against Type 14 Variants

	Type 14/46 unabs.	Type 14/46 Abs. S43	Col. 3 Unabs.	Col. 3 Abs. S43	Col. 3 Abs. Col. 7	Col. 7 Unabs.	Col. 7 Abs. S43	Col. 7 Abs. Col. 3	Col. 7 Abs. Co. 8199	Co. 8199 Unabs.	AD309 Unabs.	AD309 Abs. S43
	Precipitin tests											
M extracts												
Type 14/46	++++±	+++	++++	+++	+++±	+++	++±	−	++±	++±	++±	++
AD309	+	−	+++	++	−	+++	+++	−	+++±	−	+++	++
Col. 3	++++±	+++	++++	+++±	+++±	+++	++±	−	+++	+++±	++±	++
Col. 7	±	−	+++	++	−	+++	++±	−	+++	−	+++	++
Co. 8199	+++	+++	++++	+++	+++±	+	−	−	−	+++	−	−
	*Bactericidal tests**											
Strains												
Type 14/47	+		+			−					−	
AD309	−		+			+					+	
Col. 3	+		+		+	−	−	−		+	−	
Col. 7	−		+		−	+	+	−	+	−	+	

* + means that growth of the test strain was inhibited, compared to growth in normal rabbit serum control.

Studies were therefore undertaken to ascertain whether the precipitating antigens of Colonies 3 and 7 were M antigens by this criterion.

The results are given in Table V, and in summary form in Table IV. Unabsorbed colony 3 antiserum contained bactericidal antibodies for both colony 3 and colony 7 organisms, indicating that colony 3 possessed an M antigen shared by colony 7. Unabsorbed colony 7 antiserum, which reacted in precipitin tests with extracts of both colony 3 and colony 7 organisms, had a bactericidal effect when colony 7 cocci were used as the test strain but not when colony 3 cocci were so used. This result would be expected if colony 7 possessed, as postulated, a single M antigen, shared by colony 3, and colony 3 possessed an additional M antigen, not present in colony 7. Thus, even though the antibodies in the colony 7 antiserum reacted with the corresponding M antigen on colony 3 organisms, the possession by the latter of an additional M antigen for which no antibodies were present in colony 7 antiserum would enable the colony 3 cocci to grow out.

The reactions of absorbed sera in bactericidal tests were in accord with the scheme proposed above for the antigenic composition of the colony 3 and

colony 7 variants. Absorption of colony 3 antiserum with colony 7 organisms removed the bactericidal activity of the serum for colony 7 cocci but not for colony 3 cocci. Absorption of colony 7 antiserum with colony 3 cells removed the bactericidal activity of the serum for colony 7 cocci.

It was concluded from the above observations that the Colony 7 variant contained one M antigen which was also present on colony 3 cocci, and that

TABLE V

Reciprocal Absorption of Colony 3 and Colony 7 Antisera

Bactericidal tests

Strain tested	Antiserum	Results							
	Colony 3								
Colony 7 cocci	Inoculum	1,020,000	102,000	10,200	1,020	265	146	44	8
	Normal rabbit serum	L	L	L	L	PL	TM	TM	960
	Unabsorbed serum	TM	224	4	8	0	0	0	0
	Absorbed once (1:3) with colony 7	L	L	L	L	288	1,224	128	328
	Inoculum				930	247	137	42	14
	Normal rabbit serum				L	L	PL	PL	1,220
	Absorbed twice (1:3) with colony 7				L	L	PL	PL	2,220
Colony 3 cocci	Inoculum	1,000,000	100,000	10,000	1,000	266	125	36	12
	Normal rabbit serum	L	L	L	L	L	L	PL	PL
	Unabsorbed serum	864	60	12	0	0	0	0	0
	Absorbed 3 times (1:3) with colony 7	TM	548	416	0	0	0	0	0
	Colony 7								
Colony 7 cocci	Inoculum	1,130,000	113,000	11,300	1,130	113			
	Normal rabbit serum	L	PL	PL	TM	1,828			
	Unabsorbed serum	PL	288	48	16	0			
	Absorbed twice (1:5) with colony 3	L	L	PL	TM	2,304			
Colony 3	Inoculum	1,010,000	101,000	10,100	1,010	101			
	Normal rabbit serum	L	L	L	L	L			
	Unabsorbed serum	L	L	L	L	L			
	Absorbed twice (1:5) with colony 3	L	L	L	L	L			

Results are reported as L (plate laked), PL (plate partially laked), TM (too many colonies to count); figures represent the total number of chains present in the rotator tubes after 3 hours' rotation. (Plate count times 4).

the colony 3 organisms had a second M antigen. This second M antigen was shown to be the currently recognized M antigen of Type 14 by testing M extracts of a series of known Type 14 strains with colony 3 serum which had been absorbed with colony 7. The reactions were all positive, and the absorbed colony 3 antiserum appeared to be identical with the Type 14 serum used for routine typing purposes.

By contrast, the antigen of colony 7, which was also shared by colony 3, appeared to be a hitherto undescribed M antigen. The colony 7 antigen was considered to be an M antigen for several reasons. The fact that it was extractable from the cells by heat and acid, and an extract so made reacted

specifically with absorbed sera was compatible with its being an M antigen. M antigens are digestible by trypsin (11) and it was shown that the colony 7 antigen in a double strength M extract was digested within 15 minutes at 37°C. by crystalline trypsin solution in a final concentration of 0.01 mg. per ml., no longer reacting with absorbed homologous antiserum. Furthermore, it was shown that this antigen was located in the cell wall where the M antigen is known to be located (11–13). This was established by disintegrating a suspension of the colony 7 cocci in a Mickle disintegrator (14, 18) for 15 minutes. The soluble material released did not react with absorbed anti-colony 7 serum, but an acid extract from the washed cell walls of the cocci did react. The Type 14 M antigen of colony 3 cells was found also to be located, as was expected, in the cell walls. But the most important criterion for establishing the nature of the colony 7 antigen was its function in the bactericidal antibody tests that have been described above. Only the anti-M antibodies are believed to inhibit growth in this test, and they inhibit the growth only of strains having the corresponding M antigen.

As has been reported above, the M antigen of the colony 7 variant did not belong to any of the currently recognized streptococcal types, and it was therefore decided to term this the M antigen of Type 51.

Accordingly, strains having only the Type 14 M antigen would be called Type 14; those having only the colony 7 M antigen would be called Type 51; and those having both antigens would be called Type 14–51.

A survey was made of 42 strains previously identified as belonging to Type 14, or thought to be related to that type, to determine how often the Type 14 and Type 51 M antigens occurred together in the same strain and how often they occurred separately. 37 of the strains possessed both M antigens. Thirteen of these appeared to have the Type 51 M antigen in relatively small amounts, because while M extracts gave good, 2 plus or better, reactions with the Type 51 antisera, crude acid extracts of the type ordinarily used for typing gave weak reactions which were considered doubtful. Four strains had the Type 51 antigen only, and one strain had the Type 14 M antigen without the Type 51 M antigen.

Several strains were of special interest, and further studies were made of them.

Type 51 Strains.—Of the four strains (or sets of strains) so far identified as belonging to Type 51, two appeared during laboratory maintenance of strains identified originally as Type 14, but now known to be Type 14–51. These were colony 7, derived from AD242/28/2 as described above, and S23 Burbank, a strain derived from S23 by numerous cultural manipulations in Dr. Lancefield's laboratory (15).

The other strains were isolated from patients, and it was of interest to know whether Type 51 strains exist in nature and are capable of causing infections. Strains AD309 and AD310 (considered as one strain) were isolated from the same patient, early and late in his infection, during a mixed epidemic of acute respiratory infection at the Bainbridge Naval Training

Center in 1952 by Dr. Horace Gezon (16), and were sent to this laboratory for study because they were highly resistant to sulfadiazine in an *in vitro* test.[1] Extracts of the strains gave faint reactions in the Type 14 serum in current use at Bainbridge but the reactions were not strong enough to warrant assignment to that type, and in this laboratory no reaction was obtained with Type 14 serum. Strain AD309 was passed serially through mice 31 times and it increased in virulence from LD_{50} of 11.8 million chains to LD_{50} of 91 chains.[2] The mouse passage strain was used for immunizing rabbits, and appropriate studies indicated that the strain belonged to a hitherto undescribed type. Work on this strain was then dropped, but when the studies of the AD242/28 variants were undertaken, it was discovered that M extracts of the strains AD309 and AD310 reacted strongly with the absorbed colony 7 serum, and conversely that absorbed antiserum against AD309/31, reacted strongly with M extracts of colony 7. Extracts of the two Bainbridge strains failed to react with colony 3 antiserum that had been absorbed with colony 7 cells, but extracts of colony 3 reacted with AD309/31 antiserum. Thus, on the basis of precipitin reactions the strains AD309 and AD310 appeared to have a specific antigen identical with that of colony 7. Studies of the several strains in bactericidal tests confirmed the identity. AD309/30 antiserum had a bactericidal effect on colony 7 cocci but not on colony 3 cocci; and colony 3 antiserum had a bactericidal effect on AD309 cocci.

Because the double nature of the M structure of Type 14 streptococci was not known at the time of isolation of the Bainbridge strains, it is not possible to be certain whether the organism originally isolated from the patient was Type 51. It may have been Type 14–51, subsequently undergoing variation to Type 51 during laboratory manipulation. The fact that strains that were isolated on two separate occasions from the patient turned out on subsequent identification to be Type 51 is in favor of the idea that they existed as Type 51 variants in the patient's throat, but this cannot be considered certain.

The mouse-passaged strain AD309/31 was virulent for mice, grew well in the bactericidal test and was highly resistant to phagocytosis in the ingestion test, there being 0 and 1 per cent phagocytosis by the neutrophils after 15 and 30 minutes rotation. In these respects it was superior to colony 7. It has been found also to be the best immunizing strain in the production of Type 51 antiserum.

Another culture found to have Type 51 M antigen but no Type 14 M antigen was initially isolated in 1948 by Rothbard and Watson (17), who took serial cultures at weekly intervals from patients with pharyngitis from the acute phase of the disease through convalescence. They found that some strains isolated during convalescence had lost their capacity to synthesize the M antigen, concomitant with a loss of ability to multiply in normal human blood. One of these series belonged to Type 14. Extracts of representative cultures from this series, Type 14 M^+ and Type 14 M^-, available to us in the dried state, all gave positive reactions with Type 51 antiserum, regardless of their reaction with Type 14 antiserum. Thus, loss of the capacity to synthesize Type 14 M occurred independently and was not accompanied by a loss of Type 51 M. Serial mouse passage of the Type 14 M^-, Type 51 M^+ culture in this series restored to it the capacity to produce Type 14 M antigen. The experience with this series of cultures illustrates again the independent functioning of the Type 14 and Type 51

[1] None of the other Type 14 or Type 51 strains in this study were resistant to sulfadiazine.

[2] It should be pointed out that although an LD50 of 91 chains appears to be a high order of mouse virulence, this strain, like colony 7, was not at peak virulence. For example, in mouse virulence tests with these strains some survivors nearly always occurred among mice injected with 10^{-3} through 10^{-8} ml. of culture. Attempts to obtain a Type 51 culture of peak mouse virulence by repeated serial passage through mice and suitable for reciprocal mouse protection tests, which were unsatisfactory with the cultures at hand, were unsuccessful.

antigens and shows that a strain having the Type 51 antigen without Type 14 M antigen may occur naturally. However, it does not answer the question whether such strains can cause acute infections since this Type 51 strain was isolated during convalescence.

Type 14/46 is the strain most often used for preparing Type 14 antiserum for typing. It is of interest to note that it actually belongs to Type 14–51. Its possession of the Type 51 M antigen is shown by reaction of its M extracts with Type 51 absorbed antisera (Table III). Antiserum prepared with vaccines of this strain, kindly supplied by Dr. Lancefield, gave strong reactions with Type 14 M extracts and very feeble reactions with Type 51 M extracts, and even these feeble reactions disappeared after a single absorption with a strain of heterologous type (Table III). This is not surprising in view of the difficulty experienced, as described above, in preparing sera containing abundant antibodies for the Type 51 M antigen when the immunizing strain also possesses the Type 14 M antigen. The Type 14 serum currently supplied by the Communicable Disease Center similarly lacks antibodies for the Type 51 M antigen.

Co. 8199 is the only strain we have encountered that possesses Type 14 M antigen without also possessing Type 51 M antigen. Absorption of colony 7 antiserum with packed cells of Co. 8199 failed to remove antibodies for the Type 51 M antigen, as tested in precipitin and bactericidal tests. When Co. 8199 organisms were used in bactericidal tests, their growth was inhibited by colony 3 antiserum but not by colony 7 antiserum (Table III).

DISCUSSION

The antigenic pattern shown by Group A streptococci is subject to considerable variation. The most constant of the surface antigens is the group carbohydrate C, but even it sometimes exists in a serologically distinct, variant form (18). A T antigen may be lacking or the same T antigen may be present in several otherwise unrelated M antigenic types (19). Strains sharing a single M antigen may have different T antigens (Type 12 M, Type 10 T and Type 12 M, Type 12 T) (20). One strain has been described with two T antigens. This is strain C203, which has Type 3 M and Types 1 and 3 T antigens (1). Type 2 T antigen has been found to occur in a Group C strain (21). The R antigen occurs in several Group A types as well as in some of the other serological groups (6, 22).

The present work is the first indication that two M antigens could be possessed by a single strain of Group A streptococcus. It has long been known that some strains possess no M antigen. Such strains have been recovered from patients during convalescence, and passage through mice has yielded organisms that produced the M antigen of the parent strain (17). Such strains have also appeared during cultivation in the laboratory (23). When a strain derived during convalescence or on artificial cultivation failed to produce the M antigen of the parent strain, the assumption has often been made that the strain produces no M antigen whatever. In some cases (24, 15), this assumption has been demonstrated to be correct as was shown when rabbits immunized with the M^- variant failed to produce type-specific antibody.[3] In other cases it has

[3] One of the strains used by Lancefield and Todd (24) was a Type 14 strain, S23. This strain exists in several variant forms, three of which were obtained from Dr. Lancefield. Two mouse virulent variants (S23/94 and S23/101) were found by us to belong to Type 14–51. The other, S23 Burbank did not have the Type 14 M antigen, but did retain the Type 51 M

been assumed without adequate investigation that such is the situation. That such an assumption may be erroneous is shown in the experience reported here with AD242/28 which proved to be a mixture of Type 14 M^+ and M^- variants, both of which produced the M antigen of Type 51, and in Rothbard and Watson's Type 14–51 strain (17) which lost the ability to produce Type 14 M antigen but retained the ability to produce Type 51 M antigen. The effect of these observations is to suggest that one should not assume that a strain is M^- until it has been shown incapable of inducing anti-M antibodies on immunizing rabbits.

A further complication in the relationships of M antigens and their antisera is that they occasionally show cross-reactions, which may not be reciprocal. Lancefield (6) has described such a situation in Type 13 and Type 48 cocci, in which the Type 48 M antigen reacts with absorbed Type 13 antiserum, but the Type 13 M antigen does not cross with Type 48 antiserum. The reaction of Type 48 extracts with both Types 13 and 48 absorbed sera is not due to the existence of two M antigens, but results from a specific immunological one-way cross-reaction. This is quite different from the situation with Type 14–51, Type 51, and Type 14 strains, because the latter two strains have completely different M antigens, and the Type 14–51 strain has both M antigens.

We are satisfied that the proposed antigenic composition of the Type 14–51 strains and their variants is the best explanation of the immunological observations. An alternative explanation that has been considered and rejected is that the culture AD242/28/8 was a mixture of Type 14 cocci with Type 51 cocci that had inadvertently been introduced as a contaminant. If this were so, it should be possible to separate the two types by subculturing single colonies in series from plate to plate. While it is easy to select variants having 14 and 51 M together and 51 M alone from the mixed parent culture, it has not been possible by such a procedure to isolate a variant having 14 M alone, although it is known that such variants occur in nature (Strain Co. 8199). It would require a remarkable coincidence of contaminations in many laboratories at many times over the years to account for the many strains previously identified as Type 14 and now shown to have the Type 51 M antigen also (37 of 42 strains).

The suspicion that variation had occurred in Strain AD242/28/8 with respect to the production of Type 14 M antigen arose from observing that the culture grew as a mixture of long chain and short chain microcolonies when grown in Type 14 antiserum. That there was a mixture of 14 M^+ and 14 M^- cocci in the culture was not suggested by studying colony form, mouse virulence or the ability to grow in human blood in the bactericidal test. The fact that

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antigen. The S23 glossy variant described in their paper was obtained by cultivating a virulent variant serially in homologous serum broth through 55 transfers. It was used for immunizing rabbits and the antisera produced contained no trace of anti-M antibodies. It appears that this strain had lost both the Type 14 and Type 51 M antigens in the course of artificial cultivation.

the mixed culture reacted similarly to the parent culture in mouse virulence and bactericidal tests shows that these tests indicate that highly virulent cocci are present, but do not give assurance that cocci of lesser virulence may not also be present. On the other hand the results of the ingestion test, when viewed in the light of the results of the bactericidal test, did suggest that the culture was mixed. As far as is known, the outgrowth of streptococci in the bactericidal test (without antibody) and low degrees of phagocytosis in the ingestion test both reflect the possession of M antigen in large amounts by the test strain, together with other elements such as the capsule. It takes only a few cocci capable of resisting phagocytosis to grow out in a bactericidal test no matter how many cocci incapable of resisting phagocytosis may also be present. But the latter cocci will appear within the phagocytes in the ingestion test. In testing many Group A streptococci in this laboratory (25) in both bactericidal and ingestion tests, the results of the two tests have almost always been in agreement; *i.e.*, when the test strain grew well in blood there was very little phagocytosis in the ingestion test and *vice versa*. Thus, when a strain grows well in human blood but is extensively phagocytized in the ingestion test, it suggests that the culture consists of a mixture of variants that differ in their susceptibility to phagocytosis.

When the Type 14-51 strain was used for immunizing rabbits, antibodies for 14 M were readily produced, but antibodies for 51 M were either not produced at all or were produced in relatively low titre. This is not too surprising, for it often happens that when a multiple antigenic stimulus is given, certain antigens will predominate. For example, Lancefield has reported (6) that in producing anti-M serum for Type 48, strains without the R antigen are better immunizing strains than those that have the R antigen. It is also known that when the antigenicity of the type-specific carbohydrate of Group B streptococci is destroyed with formaldehyde, the strain is better able to induce production of group-specific antibodies than when the type antigen is unmodified (15).

Another possibility that may be considered is that the M antigen complex of the Type 14–51 strain exists as a single substance with two radicals having Type 14 and Type 51 specificity, and that one or the other or perhaps both radicals may be lost in the course of bacterial variation. In any case they appear to be able to function independently and in this report it has been convenient to consider them separate antigens.

CONCLUSION

A strain of Group A streptococcus previously considered to belong to Type 14 was shown to have two immunologically distinct M antigens, designated Type 14 and Type 51. Most strains having the Type 14 M antigen were found to have also the Type 51 M antigen, and are considered to belong to Type 14–51. Four strains had the Type 51 M antigen without the Type 14 M antigen and one strain had the Type 14 M antigen without the Type 51 M antigen.

The failure of a variant to produce the known M antigen of the parent strain does not necessarily mean that the strain is M^-, because a second M antigen may be present as was the case in several strains described here.

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