FACTORS AFFECTING THE CHAIN LENGTH OF GROUP A STREPTOCOCCI

II. Quantitative M–Anti-M Relationships in the Long Chain Test*

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In preceding studies (1, 2) most virulent strains of Group A streptococci were found to form long chains when grown in the presence of homologous antiserum. This reaction appeared to depend upon the union of M protein and anti-M antibody at the cell surface with consequent inhibition of a metabolically active chain-splitting mechanism (3). In these initial studies, however, certain exceptional strains of virulent organisms were noted which failed to form long chains in some antisera. Furthermore, some convalescent human sera from patients infected with known serological types of Group A streptococci showed discrepancies between the bactericidal and the long chain tests for anti-M antibody.

The present study indicates that the above discrepancies can be explained most often by quantitative relationships between M protein and anti-M antibody in the system. A minute excess of either antibody or antigen can be detected by chain lengthening or shortening, respectively. The use of this system for detecting subprecipitable amounts of type-specific M protein or anti-M antibody is demonstrated.

Methods

Cultures.—The strains of organisms employed were selected from among those used in the preceding study and were cultivated and preserved in the fashion previously described (3). In view of the chain-shortening effect of serum enrichment of broth, all cultures were made in Todd-Hewitt broth to which was added rabbit or sheep serum in concentrations of 10 to 20 per cent. In experiments involving dilution of broth cultures or of culture supernates, dilutions were made with 20 per cent serum-broth to keep final protein concentration relatively high. Similarly, dilutions of human or rabbit antiserum were made in normal serum of the homologous species for the same reason.

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Antisera.—Antisera were prepared in rabbits as described by Lancefield (4). Human antisera were obtained from patients with streptococcal infections of known type who were followed as part of a controlled study in pediatric out-patients at Children's Memorial Hospital in Chicago (2). All sera were processed with sterile technique. The use of preservatives was avoided. Some sera were stored at 4°C. and others were frozen and stored at either −20°C. or at −70°C. *Long chain* and *bactericidal tests* were performed as described in the preceding report (3). The results of the long chain tests are described either as the "mean chain length" or as the "long chain index." The former represents the average number of cocci in 50 streptococcal chains. The latter is derived from the ratio of the mean chain length of streptococci grown in antiserum to that of streptococci grown in control preparations containing comparable concentrations of normal rabbit serum. The results of bactericidal tests are expressed as an index of the relative growth of streptococci in blood containing homologous anti-M antibody compared with growth in control bloods in which type-specific antibody is absent (5).

EXPERIMENTAL

Inhibition of Type-Specific Long Chain Formation by Supernates of Homologous Cultures.—In the preceding study (3) it was postulated that virulent streptococci synthesize a chain-splitting enzyme when grown in protein-enriched broth. Accordingly, supernates of 18 hour rabbit serum–broth cultures of Group A streptococci were examined for possible chain-splitting activity.

Such supernates inhibited the formation of long chains in the presence of homologous antiserum. In these experiments homologous rabbit antiserum was diluted in normal rabbit serum to keep final protein concentrations in the media constant. The highest dilution of antiserum which produced long chain growth of homologous strains was employed. One-tenth ml. of antiserum, thus diluted was added to 0.3 ml. of the supernate of an overnight broth culture of the homologous strain. One-tenth ml. of an 18 hour 20 per cent serum-broth culture was added to the mixture of antiserum and the culture supernate. After incubation for 3 hours at 37°C. counts were made of streptococcal chain length. Suitable controls were included in which normal serum-broth was substituted for culture supernate.

The results of typical experiments are shown in Table I. Organisms failed to lengthen when grown in the presence of type-specific antiserum to which supernates of homologous cultures had been added.

The inhibitory activity of culture supernates was type-specific. Supernates of heterologous cultures failed to show any degree of inhibition of the long chain reaction (Table II). The inhibitory effect of culture supernates on the long chain reaction could be demonstrated only when minimal amounts of antiserum were employed in the tests. This is demonstrated by the data in Table III. A strong antiserum was titrated against a constant amount of supernate. When antiserum was present in high concentrations, the inhibition of long chaining by culture supernates could not be detected. At antiserum dilutions of 1:10 to 1:32, significant inhibition of the long chain reaction was apparent.

These results suggested that the inhibition of the long chain reaction by

homologous culture supernates was due to neutralization of anti-M antibody. The type-specificity of the inhibition suggested further that the inhibitor was M protein, apparently present in amounts which were not detectable by the usual capillary precipitin test.

This assumption appeared to be confirmed by further experiments which showed that the inhibitory activity of the culture supernates was heat-stable

TABLE I

Inhibition of Long Chain Reaction of Group A Streptococci by Supernates of Homologous Cultures

Test organism	Culture medium		Mean chain length after 3 hr. growth
	Serum	Diluent	
T14	Normal	Serum broth	11
T14	Anti-14	Serum broth	103
T14	Anti-14	T14 supernate	7
T6	Normal	Serum broth	6
T6	Anti-6	Serum broth	102
T6	Anti-6	T6 supernate	5

TABLE II

Type-Specificity of Long Chain Inhibition by Homologous Culture Supernates of Group A Streptococci

Culture	Antiserum (1:10)	Mean chain length of cultures grown in		
		Serum broth	T14 supernate	T6 supernate
T14	T14	99	6	137
T6	T6	102	145	5
T3	T3	131	80	160
T30	T30	94	166	161

and could resist boiling for 10 minutes at pH 2, a procedure which is used to extract M protein from streptococcal cells (Table IV).

Type-specific inhibition of the long chain reaction was also produced by hot acid extracts of M protein made in the conventional manner from streptococcal cells, or by solutions of highly purified homologous M protein.[1]

Detection of Minute Amounts of M Protein by Precipitin Tests Compared with Inhibition of Two Biological Methods: The Long Chain and the Bactericidal Tests. —The above experiments suggested that a bioassay for M protein might be

[1] Kindly supplied by Dr. Hutton Slade, Northwestern University.

available which would be more sensitive than detection of M protein by precipitation with immune serum and which would have the advantage, by its specificity, of requiring neither purification of the antigen, nor absorption of the antiserum. Inhibition of the long chain reaction occurred at lower concentrations of purified M protein than could be detected by precipitation with

TABLE III

Streptococcal Long Chain Inhibition When Dilutions of Antiserum Were Titrated against Constant Concentrations of Culture Supernates

Dilution of T14 antiserum	Mean chain length of cultures grown in	
	serum broth	T-14 supernate
Undiluted	125	102
1:2	90	102
1:4	65	84
1:8	61	25
1:10	66	7
1:12	67	6
1:16	62	6
1:32	29	7
1:80	10	6

TABLE IV

Effect of Heat on Long Chain Inhibiting Activity of Culture Supernates of Group A Streptococci

Test organism	Culture medium		Diluent Heated 10 min. at	Mean chain length after 3 hr. growth
	Serum	Diluent		
T14	Normal	Serum broth	37°C.	4
T14	Anti-14	Serum broth	37°C.	100
T14	Anti-14	T14 supernate	56°C.	5
T14	Anti-14	T14 supernate	65°C.	6
T14	Anti-14	T14 supernate	100°C.	10
T14	Anti-14	T14 supernate	100°C. in acid	6

immune serum. Type-specific inhibition of the bactericidal test was also observed at concentrations of M protein which were too low to be detected by precipitation.

The relative sensitivity of the three methods for detecting M protein was shown in the following experiments:

A highly purified preparation of M protein was dissolved in saline in a concentration of 1 mg. per ml. and the same serial dilutions were employed in all three tests. Precipitation tests were made with a strong, absorbed, type-specific antiserum. Long chain and bactericidal tests

were made with the highest dilution of antiserum that produced a strongly positive test. Obviously, the sensitivity of the inhibition test for M protein depended upon the amount of antibody in the system. Accordingly, the concentration of antibody was kept at the minimum compatible with a well defined test.

As shown in Table V, concentrations of 2.5 micrograms of M protein per ml. produced definite precipitation, but 1.25 micrograms of M protein per ml. resulted in tests which were equivocal. Inhibition of both the long chain and bactericidal tests was still obvious at 0.32 microgram of M protein per ml. and

TABLE V

Assay of M Protein by Precipitin Tests and by Long Chain Inhibition and Bactericidal Tests with Group A Streptococci

M protein	Precipitins	Inhibition of anti-M antibody	
		Long chain index*	Bactericidal index‡
μg./ml.			
500	++++	1.0	1
5	+	1.0	1
2.5	+	1.0	1
1.25	±	2.4	2
0.63	0	2.4	4
0.32	0	3.8	4
0.16	0	7.4	56
0.08	0	8.4	1206
0.04	0	—	1517
Blank	0	7.2	2045

* Long chain index $= \frac{\text{Mean chain length in antiserum}}{\text{Mean chain length in normal serum.}}$

‡ Bactericidal index is an expression for inhibition of growth of streptococci in presence of homologous antibody compared with growth in control bloods in the absence of antibody. <25 = 0; 25 to 50 = ±; 50 to 100 = 1+; 100 to 200 = 2+; 200 to 500 = 3+; >500 = 4+.

in the case of the bactericidal tests, the inhibition was evident at concentrations as low as 0.16 microgram per ml.

Discrepancies in the Capacity of Virulent Strains of Group A Streptococci to Form Long Chains in Homologous Antiserum.—Early in the course of these investigations an occasional virulent strain of streptococcus was encountered which, although very rich in M protein content, did not react typically by formation of long chains in the presence of homologous antisera (1). At the time these investigations were made, some of the antisera in use were not very high in anti-M titer. Subsequently, with the use of more potent antisera, all these virulent "non-long chaining strains" have been shown to react typically by the formation of very long chains in the presence of adequate amounts of anti-M antibody.

Moreover, the strains which originally had failed to "long chain" were highly virulent for mice and had been employed in tests immediately after repeated mouse passage. When such strains were permitted to dissociate to a less virulent phase they formed long chains readily with relatively weak antisera. Apparently, highly virulent strains produced amounts of M protein in excess of the amount of anti-M antibody present in the weak antisera employed. Striking long chaining resulted when the concentration of anti-M antibody was increased. Conversely, when the amount of M protein on the streptococcal cell surface was reduced by gradual dissociation of the cultures, less anti-M antibody was required to produce long chaining. The relationship between the amount of M protein on the surface of the organism and the anti-M concentration of the media is illustrated in the representative experiments shown in Table VI. A highly virulent strain (Type 3-S628) and a moderately virulent strain (Type 3-G.L. 34488) were grown, respectively, in relatively strong

TABLE VI

Relationship of Strain Virulence of Group A Streptococci to Long Chain Formation in Strong and Weak Antisera

Type 3 strain	Phase	Long chain index	
		Strong antiserum (T3-A5)	Weak antiserum (S583
S628	Highly virulent	9.0	1.5
G.L. 34488	Moderately virulent	11.0	5.4

(T3-A5) and relatively weak (S583) homologous type antisera. It is apparent that the highly virulent strain, containing excessive amounts of M protein, failed to long chain in the weak antiserum. In strong antiserum long chains were formed readily. The less virulent organism formed long chains in both antisera.

Discrepancies between the Long Chain and the Bactericidal Tests for Anti-M Antibody.—It was considered of importance to determine whether occasional discrepancies between the long chain and the bactericidal tests were due to qualitative differences in the antibodies measured by each system, or to quantitative differences in the sensitivity of the two methods. In previous studies (2) the long chain and bactericidal tests correlated well in detecting anti-M antibody in patients convalescent from Type 12 and Type 3 streptococcal pharyngitis. After 1 or more years of follow-up anti-M protein appeared to have disappeared in about 40 per cent of patients (6). In these patients, as titers declined, occasionally the long chain test became negative while the bactericidal test remained positive. The discrepancy was interpreted to reflect the greater sensitivity of the bactericidal test, inasmuch as a smaller inoculum

of streptococci was required for the latter. It was assumed that when anti-M antibody declined in titer to critical levels, a dissociation of the two tests occurred.

The relative sensitivity of the two tests, and the relationship of anti-M titers to the virulence of the strains employed, were particularly evident in long term follow-up studies of the persistence of Type 12 anti-M antibody. Because Type 12 stock cultures dissociate so rapidly, frequent mouse passage was necessary and some sera were often retested with the same strain in various phases of virulence. Occasionally sera of relatively low anti-M titer showed positive bactericidal tests and negative long chain reactions when the test or-

TABLE VII

Effect of Strain Virulence on the Sensitivity of Long Chain and Bactericidal Tests

Serum tested	Tests made with T12 streptococci			
	Moderately virulent T12/SF42		Highly virulent T12/SF42	
	Bactericidal index	Mean chain length	Bactericidal index	Mean chain length
Controls				
Normal human	—	9	—	3
Rabbit anti-12	2960	107	11,100	65
Human anti-12*				
E. P. (weak)	2960	45	360	7
Gr. (weaker)	740	18	1	6
G. W. (weakest)	7	7	1	5

* Convalescent sera of patients with Type 12 pharyngitis.

ganism was in a highly virulent phase. When the same low titer human serum was retested with the same strain in a phase of diminished virulence (a broth stock culture refrigerated 3 to 4 days) the long chain test became positive (Table VII). It appeared that discrepancies between the long chain and the bactericidal tests were encountered most often when anti-M titers were very low and when strains of very high virulence were employed.

Non-Specific Inhibition of the Long Chain and Bactericidal Tests by Some Immune Sera.—Occasionally discrepancies between the long chain and bactericidal tests were encountered which could not be explained by quantitative variations in the M–anti-M system. This was encountered most often when aged immune sera (stored at 4°C. or frozen at −20°C. or at −70°C.) were tested.

Examples of such discrepancies are shown in Table VIII. Rabbit Type 30 antiserum R-3010A showed consistently positive bactericidal tests with a

variety of Type 30 strains but never induced long chain formation. Conversely, rabbit serum R-210 produced marked long chaining but weak bactericidal tests. The latter serum originally had produced strong bactericidal tests prior to storage at −70°C. for 2 years. Anticomplementary activity of R-210 serum could not be demonstrated to account for the loss of opsonic activity.

TABLE VIII

Dissociation of the Streptococcal Bactericidal and Long Chain Tests in Some Rabbit Antisera

T-30 strains	T-30 rabbit antisera			
	R-3010A serum		R-210 serum	
	Long chain index	Bactericidal index	Long chain index	Bactericidal index
D24	1.9	3,776	24	26
D24-10	1.3	2,418	34	5
C603	1.0	1,482	26	6
C576	1.2	9,792	39	34

TABLE IX

Inhibition of the Streptococcal Bactericidal Test by R-210 Serum, and of the Long Chain Test by R-3010A Serum

Serum dilution	Tests for Type 30 anti-M antibody			
	Long chain index		Bactericidal index	
	R-210 serum diluted with		R-23010A serum diluted with	
	Normal serum	R-3010 A serum	Normal serum	R-210 serum
0	26	—	2,706	—
1/2	10	10	953	7
1/4	7	4	24	0
1/8	5	1	—	—

By mixing these two sera in various dilutions, interference with long chaining and bactericidal activity, respectively, was demonstrated. R-210 produced five-fold increase in chain length when diluted one-eighth in normal rabbit serum. When diluted one-eighth in R-3010A serum, long chaining was completely inhibited. Conversely, R-3010A serum produced positive bactericidal tests at dilutions of one-half and one-fourth in normal serum. When diluted the same way in R-210 serum, the bactericidal test was inhibited (Table IX).

Relationship of R Antigen and Anti-R Antibody to the Long Chain Reaction.— Although most discrepancies between the long chain and bactericidal tests could be explained by quantitative variations in M–anti-M relationships, or

by non-specific factors interfering with either system, there remained occasional unexplained differences in the two tests when the sera of some patients with Type 3 infections were studied. For example, one patient's convalescent sera consistently showed strongly positive long chain tests and weakly positive bactericidal tests.

The possibility was considered that such discrepancies might be due to interaction of R antigen in the test strains with anti-R antibody, inasmuch as Type 3 strains have been shown to contain this antigen frequently (7). Moreover, positive long chain tests were observed when strains containing Type

TABLE X

Relationship of M and R Antigens to Long Chain Formation in Type 3 Antisera by Type 3 Variants of Group A Streptococci

Type 3 Group A strains*	Antigenic composition‡	Mean chain length when grown in antisera			
		Unabsorbed anti-M and anti-R	Absorbed anti-M	Absorbed anti-R	Normal rabbit serum
D58X/11/12	$M^+ R^+$	158	63	57	7
S2452	$M^+ R^-$	114	55	38	7
D58X	$M^- R^+$	47	11	27	6
F208	$M^- R^-(?)$	19	14	28	5

* All strains produced large capsules when grown 2 to 3 hours in serum broth.

‡ Presence of antigens determined by precipitin tests made with hot acid extracts tested against absorbed antisera.

28 R antigen were tested with Type 28 antiserum. The latter was known to contain anti-R rather than anti-M antibody (8).

Experiments were made, therefore, with Type 3 strains of known M and R antigen content which were grown in the presence of absorbed anti-M and anti-R rabbit antisera.[2] Some of these experiments are summarized in Table X. Strains which lacked M protein failed to long chain when grown in specific anti-M antisera from which R antibody was absorbed. The situation was less clear with respect to the behavior of these strains in anti-R antiserum from which M antibody had been absorbed. In the anti-R sera studied, long chains were formed both by R-positive and by the presumably R-negative strains studied. All Type 3 strains tested grew long in unabsorbed antisera which contained both anti-M and anti-R antibodies. Very much longer chains were formed, however, by strains containing M protein. The M-negative R-positive strain formed chains of intermediate length whereas the M-negative strain, which was thought to contain either no R antigen, or at most only traces,

[2] Kindly supplied by Dr. Rebecca Lancefield.

formed chains which were slightly but significantly longer than the control preparations of the same organism grown in normal serum (Table X).

DISCUSSION

The amount of antibody required to produce a positive bactericidal or long chain test is very small indeed. In the case of the former, the largest inoculum of organisms rarely exceeds 500 streptococcal chains. As the test is performed in this laboratory, 0.05 ml. of antiserum dilution is added to each culture dilution. Therefore, no more than 10,000 streptococcal units require opsonization by 1.0 ml. of antiserum. Under the conditions employed for the long chain test, the smallest inoculum found to be practical was 50,000 streptococcal chains per ml. of antiserum (10,000 units in 0.2 ml. of antiserum). The somewhat greater sensitivity of the bactericidal test is very likely related to this difference in size of inoculum.

The striking type-specificity of the inhibition of both biological systems by crude preparations of M protein provides a further advantage of these methods over precipitation techniques. The demonstration of M protein in Group A streptococcal culture filtrates is an example of how trace amounts of this antigen may be assayed without its separation or purification from the medium in which it appears. The relative resistance of M protein to acid hydrolysis aids in extraction of this antigen from cells or tissues. Residual traces of M protein within polymorphonuclear leucocytes following phagocytosis of streptococci have been demonstrated in this laboratory employing the method of specific inhibition of the long chain test (9). This method may hold promise for tracing this antigen in tissues.

The confusion concerning M-rich strains of streptococci which failed to form long chains in homologous antisera (1) appears to be resolved by the experiments reported here. In earlier studies strong antisera were diluted to conserve their supply, or weak rabbit antisera were employed undiluted when they appeared to contain sufficient anti-M antibody to produce positive bactericidal tests and positive long chain tests with the original indicator strains of streptococci. It is now apparent that some of these sera actually contained very small concentrations of anti-M antibody which were insufficient for the excessive amounts of M protein produced in cultures of very virulent strains. The latter strains had been passed repeatedly through mice in an attempt to demonstrate long chaining in antiserum, whereas the opposite procedure, namely dissociation to a less virulent phase by unfavorable growth conditions, actually would have achieved the desired result. It is now clear that all virulent, M-containing strains form long chains in the presence of adequate amounts of anti-M antibody.

Similarly, the detection of very small concentrations of anti-M antibody in human sera sometimes requires the use of strains which are not excessively rich in M protein. This has been demonstrated recently by Lancefield (10) in studies

of the persistence of type-specific antibody in man. The great majority of patients with untreated Group A streptococcal pharyngitis develop anti-M antibody detectable by both long chain and bactericidal tests (2). When these titers fall to very low levels in some patients the greater sensitivity of the bactericidal tests becomes apparent.

Detection of very small concentrations of anti-M antibody by these methods assumes importance in studies of human vaccination with streptococcal fractions containing M protein. Failure to demonstrate anti-M precipitins following such procedures does not preclude the presence of anti-M antibody. Many mild, natural infections with Group A streptococci studied in this laboratory have resulted in very low titers of anti-M antibody which have disappeared, or virtually disappeared, within 2 years following the pharyngitis. In preliminary studies (11) multiple, small booster injections of streptococcal cell wall vaccines administered to such patients often recalled anti-M antibody to its original titer. Such low levels of anti-M antibody may still afford the host considerable resistance to reinfection with the homologous type of streptococcus.

Inhibition of streptococcal chain scission appears to be extremely sensitive to M–anti-M interaction at the cell surface. The combination of other streptococcal surface antigens with their antibodies, under appropriate conditions, also may inhibit chain scission. From the preliminary studies of the R–anti-R system reported, this surface antigen would appear to operate in similar fashion to M protein inasmuch as M-negative, R-positive strains formed long chains in homologous antisera. The results presented are not entirely clear in that strains thought to be R-negative still showed some increase in chain length when grown in anti-R antiserum. Possibly, however, amounts of R antigen too small to be detected by precipitin tests were actually present in these strains and accounted for their behavior. Further studies will be necessary to clarify this problem and to determine the relationship of the T–anti-T system (12) to inhibition of chain scission.

In the case of the highly virulent streptococci employed in these studies, however, the M–anti-M system appears to be the major determinant of long chain formation by homologous antisera. The effect of antibodies to R and T antigens would most likely be apparent with degraded variants which have lost M protein but which have retained other antigenic surface components.

SUMMARY

Minute amounts of M protein were detected in culture supernates of virulent Group A streptococci by type-specific inhibition of the long chain and the bactericidal tests for anti-M antibody. The amount of M protein that was detected by the inhibition of these biological systems was less than could be demonstrated by precipitation tests.

All strains of streptococci rich in M protein which were studied formed long

chains when grown in sufficient concentrations of anti-M antibody. Very low concentrations of anti-M antibody escaped detection by the long chain test when strains of excessive M protein content were employed. Under such conditions the bactericidal test detected anti-M antibody more sensitively than the long chain test owing to the smaller inoculum employed in the former method.

The scission of streptococcal chains may be inhibited by union of antibodies with surface antigens other than M protein. Long chains were formed when M-negative, R-positive strains were grown in sera containing anti-R antibody.

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