EVALUATION OF THE "LONG CHAIN REACTION" AS A MEANS FOR DETECTING TYPE-SPECIFIC ANTIBODY TO GROUP A STREPTOCOCCI IN HUMAN SERA*‡

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Much evidence supports the concept that immunity to Group A streptococcal infection is type-specific; that is, that recovery from infection with one type of Group A streptococcus results in immunity to that type only. The M protein antigen in Group A streptococcal cell walls determines the serologic type specificity of various strains and is an important factor associated with strain virulence. Antibody to the M protein plays a fundamental role in immunity to Group A streptococcal infection and is, indeed, the only antibody known at present to confer significant protection against this organism (1–6).

Despite the importance of anti-M antibody, extensive studies of its behavior in human streptococcal infections have been seriously hampered by the difficult and laborious biological methods heretofore available for its detection in human sera (7, 8). Recently certain virulent strains of Group A streptococci were shown to form very long chains when grown in rabbit antisera containing homologous type anti-M antibody (9). This "long chain reaction" was found to be highly sensitive and type-specific.

The present study is an evaluation of the "long chain reaction" as a biological test for type-specific antibody in sera of patients with streptococcal infections of known types. The results indicate that this readily demonstrable phenomenon correlates closely with the classical bactericidal test for anti-M antibody when appropriate indicator strains of streptococci are employed.

Materials and Methods

Strains of Streptococci.—Several strains of virulent Group A streptococci representing a wide variety of serological types were screened for the property of long chain growth in the presence of homologous type rabbit antiserum. Most of the strains studied were isolated from the throats of patients at Children's Memorial Hospital, Chicago, Northwestern Uni-

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versity Medical School Clinics, and Great Lakes Naval Training Center.[1] In addition, some well known laboratory strains maintained in a state of high virulence by frequent mouse passage also were studied.[2]

As originally reported (9), the strains that showed most striking long chain growth in homologous type antiserum were highly virulent and very rich in M protein. Dissociation to a less virulent phase as a result of repeated broth passage, or of prolonged storage in broth stock cultures, resulted in a loss of the long chaining effect. It also was noted, however, that not all virulent streptococci formed long chains in homologous antiserum with equal readiness despite their content of large amounts of M protein. The reason for this strain variation is not yet clear. Fortunately, however, appropriate variants sensitive to the long chain effect of homologous type anti-M antibody can be found as indicator strains for all serologic types tested so far (Types 1, 3, 4, 5, 6, 12, 13, 14, 19, 30, and "Red Lake" strain (10)).

Long chaining indicator strains were preserved by lyophilization or by quick freezing equal parts of a young (8 hour) broth culture and defibrinated sheep's blood in a dry-ice alcohol mixture and subsequently storing at −70°C. Stock cultures were prepared by inoculation of Todd-Hewitt broth with lyophilized or frozen cultures. After one or two broth passages to stimulate active growth, an 18 hour broth culture was stored at 4°C. Overnight growth of broth cultures made from these stocks were used for each experiment. Most strains remained fairly stable in refrigerated stock cultures for about 2 weeks. However, on repeated broth passages some strains showed a tendency to dissociate into non-long chaining strains. This was particularly true of all strains of Type 12 studied and of the "Red Lake" strain. Frequent mouse passages were required to maintain both virulence and long-chaining properties.

Antisera.—Antisera against various types of streptococci were prepared by injecting rabbits repeatedly intravenously with suspensions of heat-killed streptococci according to the method of Lancefield (11). Assays for type-specific antibody in these sera were made by a modification of the bactericidal test (see below). Pools of rabbit antisera prepared for each serological type were employed as routine "positive controls" in all assays of human sera for type-specific antibody. The rabbit sera were processed with sterile technique and were stored without preservatives at 4°C.

The Long Chain Test.—To test tubes 9.0 x 100 mm. were added 0.2 ml. of the serum to be tested for type-specific antibody and 0.05 ml. of a 10^{-2} broth dilution of a 16 to 18 hour broth culture. Tubes containing normal rabbit serum instead of the test serum were included in all experiments as "negative controls." In addition, a "positive control" tube containing strong homologous type rabbit antiserum was included with each run of unknown sera. To conserve antiserum of known high titer, dilutions of one part of serum to five parts of broth were made and 0.2 ml. of the diluted serum was added to the test tubes. The final concentration of serum in the test should be at least 20 per cent to insure optimal growth conditions and to prevent spontaneous formation of long chains which may occur in media of low protein content (see below). The tubes were closed with sterile rubber stoppers and were incubated in a water bath at 37° for 3 to 4 hours. The tubes were held stationary during the incubation period rather than rotated as described in an earlier report (9). Longer chains were observed when agitation was minimal. After mixing by gentle inversion of the tubes, a drop of culture was placed on a microscopic slide, covered with a glass coverslip, and examined immediately under the microscope. With proper lighting a stain was unnecessary. The number of cocci in each of 50 chains, selected at random from several representative fields, was counted and the mean chain length of the population calculated. The mean chain length

[1] Kindly supplied by Mr. Paul Frank, Naval Medical Research Unit No. 4, Great Lakes, Illinois.

[2] Kindly supplied by Dr. Rebecca Lancefield, The Rockefeller Institute, New York.

of cultures grown in human serum tested was divided by the mean chain length of cultures grown in normal rabbit serum as a control. This ratio was called the "long chain index."

For a suitable test the strain employed should grow in short chains (mean length of 4 to 12 cocci) in normal serum and should show a mean increase of at least five to ten times or more when grown in a strong homologous rabbit antiserum. The chain length of virulent chains of streptococci is usually short in protein-enriched media and tends to become longer spontaneously in plain Todd-Hewitt broth (10). It is essential, therefore, that the control tubes contain normal serum or other suitable protein solutions as enrichment media. The pH of the media also should be adjusted carefully to a range of 7.5 to 8.0 before inoculation of cultures. Strains that fail to grow short under these conditions should be suspected of having undergone dissociation even though they remain typable by conventional serological tests. They often may be restored to their former phase of virulence by mouse passage. Sera that produce a threefold increase in the mean chain length of streptococci compared with the mean chain length of cultures grown in normal rabbit serum were considered to be positive for type-specific antibody (see Results).

Bactericidal Test.—All sera assayed for type-specific antibody by the "long chain" test also were studied by the bactericidal test employing a recent modification of the latter method described in detail elsewhere (7). By this method the survival of streptococci in fresh heparinized human blood to which antiserum is added is compared with the survival of these organisms in the same blood to which normal rabbit serum is added as a control. In the presence of type-specific antibody, streptococci are rapidly phagocytized and destroyed. The relative growth in the test and control is compared and expressed as a bactericidal index (8). Thus, an index of 100 means that the bacterial population increased 100 times more in the control blood than in the test blood. As a measure of type-specific antibody an index of 25 to 50 is doubtfully positive (±); 50 to 100 is weakly positive (1 plus); 100 to 200 is 2 plus; 200 to 500 is 3 plus; and greater than 500, is 4 plus.

Human Sera.—The human sera tested in this study were obtained from patients with pharyngitis due to Group A streptococci of Types 12 and 3. These patients were followed as part of a large scale controlled study in pediatric out-patients now in progress at Children's Memorial Hospital in Chicago. The details of the type-specific immune response in these patients will be presented in a subsequent report (12). An additional group of sera[3] from patients with glomerulonephritis, which were known to contain Type 12 antibody, were also tested for the long chain reaction.

All sera were processed with sterile technique and stored at 4°C. The use of preservatives was avoided since such agents markedly inhibited streptococcal growth and prevented long chain formation.

RESULTS

Type-Specific Long Chain Growth of Group A Streptococci in Human Immune Sera.—In preliminary experiments patients known to have sustained pharyngeal infections with Type 12 Group A streptococci were bled and their sera were studied for Type 12 anti-M antibody by the bactericidal test. Sera were selected that produced the strongest and weakest bactericidal tests, respectively, against Type 12 organisms. Strains of Type 12 previously found to grow in long chains in the presence of rabbit antisera of homologous type, were grown for 3 to 4 hours in media containing the human immune sera.

[3] Kindly supplied by Dr. David P. Earle, Jr., Northwestern University Medical School, Chicago.

In general, it was found that human sera strongly positive for type-specific antibody by the bactericidal test almost invariably produced a striking increase in streptococcal chain length. The mean increase in length ranged from three to fourteen times that of the same strain grown in normal rabbit serum. The average increase in length in strong antisera was nine times that of the controls (Table I). Sera weakly positive for anti-M antibody by the bactericidal test produced, in general, a less marked increase in chain length (Table I). It was unusual, however, for any serum weakly positive by the bactericidal test to

TABLE I

Correlation of Positivity of Bactericidal and Long Chain Tests in Convalescent Sera of 43 Patients with T12 Group A Streptococcal Infections

No. sera	Bactericidal test	Long chain tests			
		Mean* ± S.D.‡		Ratio: test to control§	
		Test	Control‖	Mean¶	Range
12	++++	96 ± 23	10 ± 3	9.2	3–14
3	+++	73 ± 13	6 ± 1	6.0	5–7
4	++	49 ± 35	9 ± 1	5.0	3–12
6	+	40 ± 13	9 ± 3	4.8	3–6
18	0	14 ± 3	10 ± 3	1.4	0.5–2.5

* Average of the mean chain length of individual tests.

‡ S.D., standard deviation.

§ Ratio of the mean chain length of streptococcal grown in antiserum divided by the mean chain length of streptococci grown in normal rabbit serum is referred to as the "long chain index" in the text.

‖ Mean chain length of streptococci grown in normal rabbit serum is determined as a control for each test serum.

¶ Mean of the ratios of the individual tests.

produce less than a threefold increase in chain length. Chi square analysis of the differences of the chain length of a population of streptococci grown in human antisera, compared with that of a control population of streptococci grown in sera free of antibody, indicated that a threefold or greater difference in mean chain length was highly significant ($p = < 0.01$). Chi square analyses were employed in tests for statistical significances of population differences in chain length because the distribution curves of the chain length of populations of streptococcal cultures grown in antisera were often skewed rather than normal. This was due, presumably, to fragmentation of the longer chains, although it also could have represented some variants in the population that failed to increase significantly in length.

Type Specificity of the Long Chain Reaction in Human Sera.—All sera that

gave a positive long chain test with Type 12 organisms were also tested against Type 14 and Type 30 organisms. All failed to produce a threefold increase in chain length with these heterologous types. In addition, none of the 18 sera that were negative for Type 12 antibody produced a significant increase in chain length against any of the three types tested.

The type specificity of the long chain reaction for anti-M antibody in unabsorbed human sera was tested further in experiments employing five strains of streptococci of different serological types (Types 1, 3, 6, 14, and 30).

The strains representing these types were all previously shown to grow in long chains in the presence of homologous type rabbit antisera. Each of these strains was tested against five unabsorbed sera which were known to contain anti-M antibody to one of the five sero-

TABLE II *a*

Indices of Bactericidal Tests Made with 5 Types of Streptococci against Each of 5 Unabsorbed Immune Sera*

Antisera	Serologic types of cultures				
	T12	T3	T14	T6	T30
Human T12.....	**3140**	4	0.7	0	1
Human T3.....	2	**272**	2	0	1
Rabbit T14.....	2	0	**2625**	0	1
Rabbit T6.....	2	1	2	**9750**	1
Rabbit T30.....	2	0	3	0	**8300**

* 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; 25–50 = ±; 50–100 = 1+; 100–200 = 2+; 200–500 = 3+; >500 = 4+.

logical types of streptococci. Two of these antisera were obtained from patients convalescent from Type 12 and Type 3 streptococcal pharyngitis, respectively. The other three antisera were obtained from rabbits immunized with heat killed vaccines of types 6, 14, and 30, respectively.

The data presented in Table II *a* demonstrate the high titers of homologous type antibody that were found in each serum tested against each of the five streptococcal strains by the bactericidal test. The clear type specificity of the bactericidal test system is indicated by the absence of a positive test in any heterologous system. The bactericidal activity is expressed as an index of suppression of growth of streptococci in blood containing anti-M antibody according to a formula derived in previous studies (see Materials and Methods). The assay for type-specific antibody was repeated employing the same five strains of streptococci and the same antisera in the long chain test. An increase in mean chain length three times or greater than that observed in control cultures, grown in the presence of normal rabbit serum, was observed only in the five

homologous type tests. In none of the twenty tests made with heterologous type sera was a threefold increase in chain length observed (Table II *b*).

Absorption of the sera with streptococcal cells of homologous type removed the long chaining effect, whereas similar absorption of sera with streptococci of heterologous type failed to do so.

TABLE II *b*

Chain Length Indices of 5 Types of Streptococci Grown in the Presence of Unabsorbed Immune Sera*

Antisera	Serologic types of cultures				
	T12	T3	T19	T6	T30
Human T12.....	**4.5**	1.2	1.3	0.8	1.5
Human T3.....	1.1	**4.2**	1.2	0.8	1.0
Rabbit T14.....	0.9	1.4	**28.3**	2.2	1.8
Rabbit T6.....	0.7	1.1	1.2	**13.0**	0.8
Rabbit T30.....	0.8	2.0	0.8	1.0	**18.4**

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

TABLE III

Long Chain and Bactericidal Tests Made on Sera of a Patient Convalescing from T12 Streptococcal Pharyngitis

Days after infection	Bactericidal index*	Long chain index* with strains		
		T12	T14	T30
0	0	—	—	—
21	0	0.8	1.8	0.8
35	5	3.8	1.4	2.1
60	810	4.3	0.5	0.9
170	900	6.2	1.2	1.5

* See footnotes to Tables II *a*, and II *b* for explanation of indices.

Demonstration of the Appearance of Type-Specific Antibody by the Long Chain Test in Patients Convalescent from T12 Streptococcal Pharyngitis.—Studies were made on the sera of children who were bled serially following either treated or untreated pharyngitis due to either T12 or T3 streptococci. The long chain test was compared with the bactericidal test for the detection of the appearance of type-specific antibody during convalescence.

A sample protocol of a patient with Type 12 pharyngitis is shown in Table III. This patient developed a high titer for type-specific antibody by the 60th

day following the infection. The titer remained high in the serum obtained on the 170th day of convalescence. Parallel studies were made employing the long chain test. An increase in the chain length of homologous Type 12 organisms was apparent in tests made at 35 days and was still more apparent in tests made at 60 and 170 days after the infection. No significant increase in chain length of heterologous Types 14 and 30 streptococci was observed when these strains were grown in the same sera.

Similar results were demonstrated in patients convalescent from untreated, mild T3 streptococcal pharyngitis. A representative experiment is shown in Table IV. Again, the bactericidal test which was initially negative became strongly positive on the 60th and 90th day after the infection. The long chain test showed a markedly positive reaction at the same times. Control tests

TABLE IV

Long Chain and Bactericidal Tests Following T3 Streptococcal Pharyngitis

Time after infection	Bactericidal index*	Long chain index* with strains		
		T3	T14	T12
days				
0	7	2	—	—
60	1740	6	2	0.3
90	1160	7.8	1.3	0.4

* See footnotes to Tables II *a*, II *b* for explanation of indices.

made with the same sera against heterologous Type 12 and 14 strains were negative.

A Comparison of the Relative Effectiveness of the Bactericidal and the Long Chain Test for Detection of Type-Specific Antibody in Human Sera.—A comparison of the bactericidal and the long chain tests made with acute and convalescent sera of patients in this study is shown in Table V. Of 217 sera obtained from 99 patients and studied with both tests, 66 were positive by the bactericidal test and 63 by the long chain test. There were 151 sera negative by the bactericidal test and 154 negative by the long chain test. Only 11 of the 217 sera failed to show correlation. Seven of the 66 that were positive by the bactericidal test failed to show a positive long chain test; and 4 of the sera negative by the bactericidal test were positive by the long chain test. None of the sera that were positive by either test showed cross-reactions with two heterologous strains included as controls, Types 14 and 30. The results show close agreement and almost equal sensitivity of the two tests.

In most of the eleven sera that failed to show correlation the antibody appeared to be present in relatively low concentration. A few sera were

encountered, however, which were strongly positive by the bactericidal test but which failed to produce long chain growth upon repeated testing. The reverse situation was also encountered occasionally. The reason for these variations is not apparent.

The agreement of the two methods in detecting the appearance of type-spe-

TABLE V

Correlation of Long Chain and Bactericidal Tests Made on 217 Sera Obtained during Convalescence from 97 Patients with Either T12 or T3 Streptococcal Infections*

Long chain test	Bactericidal test		
	Positive	Negative	Total
Positive	59	4	63
Negative	7	147	154
Total	66	151	217

* Includes sera obtained on the 1st day of infection, at 21, 35, 60 days, or at 6 months thereafter, depending upon the duration of follow-up.

TABLE VI

Detection of Type-Specific Antibody in Convalescent Sera of Patients with T12 or T3 Infections

	T12		T3		Total	
	C*	Rx‡	C	Rx	C	Rx
No. of Patients	32	41	12	14	44	55
Per cent positive by:						
Bactericidal	63	10	67	7	64	10
Long chain	62	5	58	0	61	4
Either	66	10	67	7	66	10

* C, untreated control group.

‡ Rx, group receiving penicillin treatment.

cific antibody in the treated and the untreated groups of patients is shown in Table VI. Of 44 patients in the control group (untreated) 66 per cent developed detectable antibody to the infecting type of streptococcus. Sixty-four per cent were detected by the bactericidal test and 61 per cent by the long chain test. Of the 55 patients who were treated with penicillin, only 10 per cent had detectable antibody; 10 per cent by the bactericidal test and 4 per cent by the long chain test.

DISCUSSION

The bactericidal test has been generally regarded to be the most useful method for the detection of type-specific streptococcal antibody (6–8, 13). Although itself quite difficult and laborious, it is much more practical for extensive studies made with human sera than is the mouse protection method which requires large amounts of sera and is expensive and laborious (14).

Precipitin tests for anti-M antibody in human sera suffer from uncertainties in the interpretation of the specificity of the precipitates formed (15). This is due primarily to the problems involved in the purification of the M protein antigen and for establishing criteria for purity (16). Moreover, sera that contain either no precipitating antibody, or at most, traces of precipitate, when tested with extracts containing M protein, may be shown to produce relatively strong bactericidal tests. The precipitin system, therefore, does not appear to be as sensitive or as readily interpretable as the bactericidal test system.

Complement fixation methods with antigens prepared from crude extracts of streptococci require absorption of human sera with heterologous strains to remove cross-reacting non-specific streptococcal antibodies (17). Such absorptions frequently reduce specific antibody titers, particularly when the latter are present in low concentrations, and often fail to eliminate cross-reactions with heterologous types of streptococci. Agglutination (18) and hemagglutination (19) methods suffer from the same problem of cross-reactions.

The relative simplicity of the long chain reaction and its striking specificity and sensitivity would appear to make it a valuable addition to the methods available for the study of type-specific immunity, particularly for screening studies of large numbers of human sera.

Despite its apparent simplicity, however, certain limitations of the long chain method should be emphasized. Although the long chain reaction appears to be closely related to the M-anti-M system the mechanism of the reaction is not clear. Some strains rich in M protein do not form long chains readily with all homologous antisera (8). Conversely, the failure of an occasional serum (demonstrated to contain anti-M antibody by the bactericidal test) to produce a long chain reaction with some ordinarily good indicator strains is also not understood. The possibility exists that the antibodies involved in the two systems are not identical or that some inhibiting substance in certain sera may interfere with the complex mechanism involved in one or the other biological systems employed.

Of major importance in the long chain test is the requirement of indicator strains that are carefully maintained in the phase of high virulence. Some strains dissociate rapidly to less virulent phases when they are grown or stored on artificial media. Such dissociation leads to loss of M protein and thus to failure to increase in chain length in the presence of anti-M antibody. For this

reason it is useful to employ mouse virulent strains, maintained in the proper phase by occasional mouse passage.

The sera tested also must be free of substances that inhibit the growth of streptococci, such as antibiotics and preservatives, and must be free of contaminants. Penicillin in serum may be removed by treatment with penicillinase[4] without interfering with either the bactericidal or the long chain tests (10).

Finally, interpretation of the long chain test is aided considerably by including as a "positive control" a standard serum of known high homologous anti-M antibody titer. With strict observance of the above precautions the long chain test should prove to be a useful method for measuring human type-specific antibody.

SUMMARY AND CONCLUSIONS

Certain strains of Group A streptococci showed striking increase in chain length when grown in liquid media to which was added human sera that contained antibody to M protein of homologous type. This "long chain reaction" was shown to be a highly specific and sensitive biological test for human type-specific antibody and correlated closely with the classical bactericidal test.

Patients infected with Type 12 or Type 3 Group A streptococci showed the appearance of anti-M antibody in their sera by both methods at similar intervals during convalescence. Of 217 sera studied in these patients the two tests showed agreement in all but 11 specimens. Of 99 patients who were bled serially following Type 12 or Type 3 infections, and whose sera were tested by both methods, there was close agreement, the bactericidal test being only slightly more sensitive.

The advantages and limitations of this new biological test for human type-specific immunity are discussed.

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