FACTORS AFFECTING THE CHAIN LENGTH OF GROUP A STREPTOCOCCI

I. Demonstration of a Metabolically Active Chain-Splitting System*

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At the close of the nineteenth century much attention was given to the effect of immune serum upon the growth of microorganisms. In some of these early studies it was noted that certain bacteria not only clumped together but sometimes formed threads or chains when grown in homologous antiserum.

Thus, in 1889 Charrin and Roget (1) commented that *B. pyocyaneus* grown in immune rabbit serum formed "chainettes" of 6 to 10 segments. Two years later Metchnikoff (2) observed that *Vibrio metchnikowii* formed *de spirilles allongée*, in addition to thick masses, when grown in immune serum and that *le microbe de la pneumonie* also grew in long chains (*des paquets de streptocoques très longs*) in the serum of vaccinated rabbits.

In 1897 Bordet (3) observed that streptococci formed long, tortuous chains in rabbit antisera. The following year "long chaining" received specific attention in the studies of Pfaundler (4) who described the phenomenon as the "thread reaction" (*Fadenbildung*). He observed the formation of long threads when strains of *E. coli* and *B. proteus* were grown in sera obtained from the same patients from whom the organisms were isolated. This was interpreted as a special form of agglutination in which organisms failed to divide along their long axes but remained as rods adherent end-to-end.

During the early part of the twentieth century several investigators studying the effect of immune serum upon the pneumococcus referred to the "thread reaction." By 1928 the sensitivity and potential usefulness of this phenomenon were appreciated by Bailey (5) who wrote: "The presence of such antibodies (. . . . agglutinins and precipitins . . . .) in very small amounts, not demonstrable by ordinary serological methods, may be revealed if the organism be grown in the presence of serum or blood (the thread reaction of Pfaundler). For example, the sera of patients with pneumonia frequently show no agglutinins by the usual methods, but if pneumococci are sown in such sera they grow out in masses with chain formation."

For the most part, however, relatively little attention was paid to this phenomenon and in the same year (1928) Pfaundler's thread reaction was considered "of historic interest only since it is essentially an agglutination reaction" (6).

Following Lancefield's serological classification of Group A streptococci into types on the basis of the surface M antigen (7), investigators sought anti-M precipitins and agglutinins in convalescent sera of infected individuals. Lack of type-specificity made interpretation of the observed reactions difficult (8). Moreover, agglutinins and precipitins frequently could not be demonstrated in convalescent human sera (9). In view of these problems biologic tests which were very sensitive and type-specific, such as mouse protection and opsonophagocytic tests with human blood (the bactericidal test), were employed to study human immunity to Group A streptococci (10).

In the course of studies employing the bactericidal test, Bordet's original observation of long chain growth of streptococci in homologous antiserum was encountered independently in this laboratory (11). It was shown that this phenomenon was dependent upon the interaction of M protein and its antibody. The long chain reaction was found useful for detecting human type specific antibody with almost the same sensitivity as the bactericidal test (12). Long chaining was also used recently by Wilson (13) to identify M protein in strains of streptococci.

The present studies will show that virulent strains of Group A streptococci when grown in the presence of anti-M protein form long chains as a result of the inhibition of a metabolically active system, presumably an enzyme or group of enzymes, which is involved in the scission of streptococcal chains.

Materials and Methods

Cultures.—Numerous strains of virulent Group A streptococci representing 10 serological types were surveyed for the property of long chain formation in homologous type antiserum. Most of these strains were freshly isolated from throat cultures of patients with acute pharyngitis at Children's Memorial Hospital,[1] The Naval Medical Research Unit Number 4,[2] and the Northwestern University Clinics. Some additional streptococcal strains were supplied to us from the laboratories of Dr. Rebecca Lancefield, The Rockefeller Institute, New York; Dr. Elaine Updyke, the Communicable Disease Center, Chamblee, Georgia; Dr. Armine Wilson, the Dupont Institute, Wilmington, Delaware; and Dr. W. Barry Wood, Johns Hopkins Medical School, Baltimore, Maryland. The strains of pneumococci employed were supplied by Dr. Colin M. MacLeod, University of Pennsylvania, Philadelphia. Most virulent strains exhibited the long chaining phenomenon. Suitable representatives of Types 1, 3, 4, 5, 6, 12, 14, 19, 30, and "Red Lake" were preserved by lyophilization in rabbit blood. Frozen stocks of some strains also were prepared by resuspending the cells sedimented from 40 ml. Todd-Hewitt 16 hour broth cultures in 3 to 5 ml. sterile rabbit blood, dispensing 0.2 ml. aliquots into serological tubes, and quick-freezing in a dry ice–alcohol bath. These cultures were then stored at −70°C. in a mechanical deep freeze.

Stock cultures were made by inoculating 5 per cent rabbit blood–Todd-Hewitt broth with stored strains and incubating for 16 hours at 37°C. After two further passages in Todd-Hewitt broth to stimulate rapid growth, the cultures were stored at 4°C. From these refrigerated stocks

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[1] Kindly supplied by Dr. Alan C. Siegel.

[2] Kindly supplied by Mr. Paul Frank.

fresh overnight Todd-Hewitt broth cultures were prepared for each day's experiments. Refrigerated stock cultures were renewed approximately every 2 weeks.

Stock cultures of most virulent strains were stable when maintained in this manner. Strains of Types 12, 4, and 49 ("Red Lake" strain) were, however, consistently unstable and frequently dissociated under these conditions. Such strains required frequent mouse passage to maintain them in a virulent state. Meticulous attention to the preservation and maintenance of the strains in a highly virulent phase was essential in this work.

Antisera.—Antisera were prepared in rabbits as described by Lancefield (14).

The Long Chain Test.—In our initial work (11) the long chain test was made simultaneously with a bactericidal test, and cultures were incubated in a roller tube apparatus employed in the latter procedure. It was obvious that agitation of the tubes was unnecessary in the long chain test, and in some cases was detrimental owing to the susceptibility of some strains to fragmentation into short chains under these conditions.

The test, as it was carried out in most experiments reported here, consisted of adding 0.3 ml. Todd-Hewitt broth to 0.1 ml. of antiserum, and inoculating this mixture with 0.1 ml. of a 10^{-2} dilution in broth of an overnight culture of the strain being studied. The tubes were closed with sterile rubber stoppers and incubated at 37°C. in a serological water bath for 3 hours. When greater sensitivity of the test was required to detect very low serum levels of antibody to M protein, higher antiserum concentration and a smaller inoculum of streptococci were employed. In studies on human serum 0.2 ml. of serum was inoculated directly with 0.05 ml. of a 10^{-2} or 10^{-3} dilution of an overnight culture (12).

Occasionally at the end of the 3 hour incubation, if growth appeared poor by visual observation, an additional hour of incubation was allowed. The cultures were mixed by gently inverting the tubes twice. Moist preparations were made by placing a drop of the culture adherent to the rubber stopper onto a clean glass slide. This was covered with a coverslip. The preparation was then observed with an oil immersion lens. The number of cocci in 50 individual chains were counted and the mean chain length calculated. Appropriate controls in which normal serum was used in place of antiserum were included in all tests. An additional "positive control" of strong homologous rabbit antiserum was included with each test. When additional ingredients were added to the test medium to study their effect on the reaction, they were dissolved in Todd-Hewitt broth, enriched with 20 per cent normal rabbit serum, and were substituted for the 0.3 ml. of broth used in the standard test. The final concentration of protein in the media was kept at no less than 20 per cent since, as will be shown below, chains of streptococci lengthen spontaneously in the absence of adequate protein in the media.

Bactericidal Tests.—The bactericidal test employed for detection of anti-M antibody was identical to that used in previous studies (15). For convenience, the results of the bactericidal tests were expressed as an index based on the relative growth of streptococci in blood containing homologous anti-M compared with control bloods in which type-specific antibody was absent (15).

Demonstration of Capsules.—Capsules were demonstrated by the wet India ink method as described by Wilson (13).

EXPERIMENTAL

Disruption of Long Chains by M Protein.—Experiments were undertaken to determine whether or not long chains formed in the presence of minimal anti-M antibody could be disrupted by the addition of M protein. It was reasoned that by addition of excess antigen a redistribution of the antibody from the chains to the soluble M protein might occur. Chains thus denuded of absorbed antibody might be expected to fragment into shorter units.

The following experiments were carried out to test this hypothesis: A mixture of 0.1 ml. of 25 per cent normal rabbit serum-broth and 0.1 of an appropriately diluted antiserum was inoculated with 0.1 ml. of a 10^{-2} dilution of an 18 hour broth culture of a virulent strain of Group A streptococci. After 3 hours' incubation at 37°C., 0.3 ml. of an M protein extract of homologous cells was added to the system. After a further incubation of 30 minutes at 37°C. the cocci in each chain were counted. As controls, extracts of heterologous M protein, and normal saline, respectively, were added to some tubes.

It was evident that upon the addition of homologous M protein extracts to long chains formed by previous growth in minimal amounts of anti-M antibody,

TABLE I

Type-Specificity of Chain Shortening of Group A Streptococci by M Protein Extracts

Culture	Serum	Mean chain length					
		Before M extract	30 min. after addition of M extracts				
			T-6	T-19	T-5	T-14	Saline
Type 6	Normal	11					
	Anti-6	108	12	80	120	—	108
Type 19	Normal	10					
	Anti-19	86	72	12	69	—	86
Type 5	Normal	10					
	Anti-5	85	75	63	11	81	85
Type 14	Normal	8					
	Anti-14	72	64	67	67	10	72

a marked reduction in chain length occurred. The chain disruption was completely type-specific (Table I). The same effect was produced when homologous purified M protein[3] was used instead of crude acid-heat extracts.

Effect of Physical and Chemical Factors upon the Disruption of Anti-M Induced Long Chains.—We had regarded the long chain reaction of Group A streptococci grown in homologous antiserum as an end-to-end agglutination of individual cocci in which absorption of antibody to the cells presumably made the chains less susceptible to fragmentation by physical forces in liquid suspensions. If this were the case, disruption of long chains by the addition of M extracts, or of purified M protein solutions, might be expected to occur after the organisms were killed by heating at relatively low temperatures. Therefore the following experiment was done:

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[3] Kindly supplied by Dr. Hutton Slade, Northwestern University Medical School.

Virulent strains of streptococci were grown in homologous antiserum for 3 hours at 37°C. The cultures were then heated at 60°C. for 15 minutes. No viable organisms remained. Solutions of M protein were added and the cultures were incubated another 30 minutes at 37°C. Control tubes of unheated cultures of streptococci grown in antiserum were included.

Under these conditions heat-killed long chains did not fragment upon the addition of excess soluble M protein. Further experiments in which the long chains were killed by mercuric chloride, formalin, or penicillin led to similar results.

These results suggested that the splitting of long chains following the addition of excess M protein might be due to an active metabolic system, possibly a chain-splitting enzyme.

Temperature and pH Optima for Chain Scission.—

Long chains of streptococci were formed by growth in homologous antiserum. Temperature and pH, respectively, were then adjusted to various levels and an excess of homologous M protein was added. After further incubation for 30 minutes chain length was determined.

It was evident that the rate of chain scission was temperature- and pH-dependent. Optima were approximately 37°C. and pH 7.5 (Figs. 1 and 2). At 0 or 50°C. there was little or no chain splitting. The reaction was also markedly inhibited at pH below 6 or above 8. These data further suggested an enzymatic mechanism for chain scission. The effect of other methods of enzyme inhibition on chain scission was, therefore, tested.

Inhibition of Chain Scission by Sublethal Concentrations of Mercuric Chloride. —The scission of long chains by excess M protein could also be inhibited by concentrations of mercuric chloride below that necessary to kill the streptococci.

Long chains of streptococci were formed by 3 hour growth in minimal concentrations of antiserum-broth and chain counts were made. Varying concentrations of $HgCl_2$ were added and the cultures incubated 10 minutes at 37°C. M extract was then added and the tubes incubated for an additional 30 minutes. Chain counts were made again.

Inhibition of chain scission was directly related to the concentration of $HgCl_2$. At concentrations of 0.1 micromole of $HgCl_2$ chain scission was completely inhibited. Partial inhibition was still apparent at a concentration of 0.001 micromole of $HgCl_2$ (Fig. 3).

The Rate of Chain Scission.—Taking advantage of the ability of $HgCl_2$ to stop the disruption of chains, it was possible to determine the rate of the reaction.

After cultures in a series of tubes had formed long chains by growth in antiserum-broth for 3 hours, M extract was added as in previous experiments, followed by the addition of an inhibitory concentration of $HgCl_2$ to each tube at 1, 3, 5, 7, and 9 minutes, respectively.

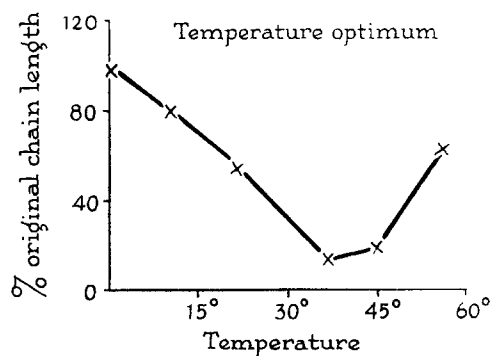The chain-splitting reaction began almost immediately upon the addition of

FIG. 1. Effect of incubation temperature on chain shortening by homologous M protein extract.
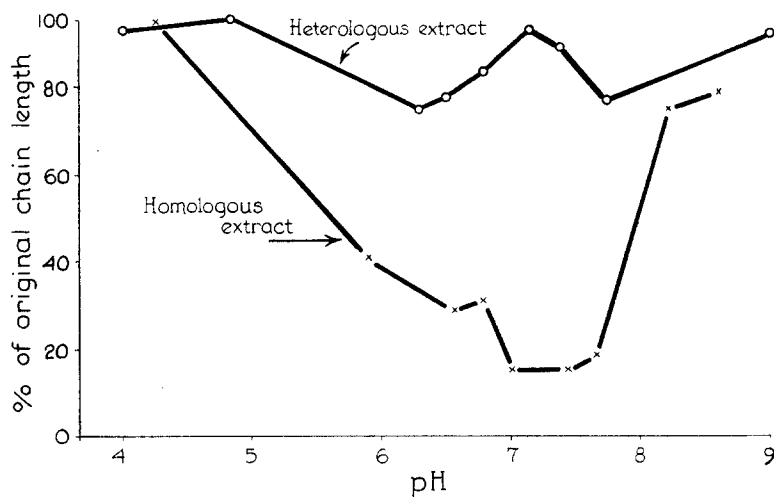

FIG. 2. Effect of pH on chain shortening by homologous M protein extract.

M protein and was essentially complete in 5 minutes. When mercuric chloride was added 1 minute following the addition of M extract, chain length was already significantly reduced, and 5 minutes after the addition of M extract to other tubes, no inhibitory effect of $HgCl_2$ could be detected (Fig. 4).

Inhibition of Chain Scission by Penicillin.—The above experiments suggested that interference with cellular metabolism of streptococci inhibited the scission of long chains. To obtain further information on this point the anti-M–induced long chains of streptococci were exposed to penicillin for varying periods of time prior to adding M protein to the cultures.
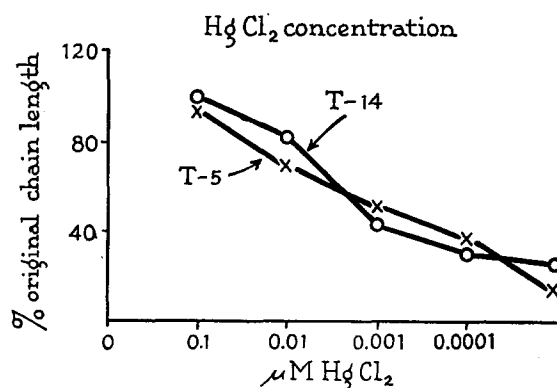
FIG. 3. Effect of mercuric chloride on chain shortening by homologous M protein extracts.
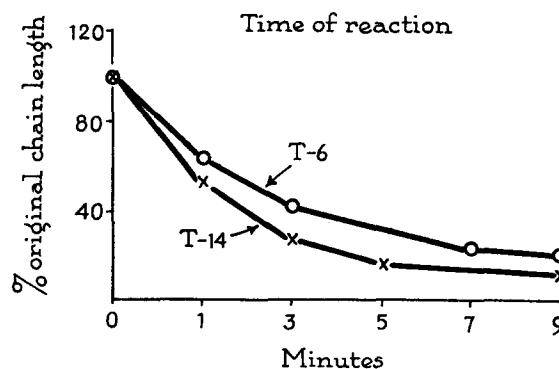

FIG. 4. Rate of chain shortening by homologous M protein extracts. Mercuric chloride was used to stop the reaction at the indicated times following addition of M extracts.

Long chains of streptococci were induced by growth for 3 hours in 0.2 ml. of homologous antiserum-broth in each of a series of tubes. One-tenth ml. of a penicillin solution containing approximately 15 units of penicillin per ml. was added to each tube. After 0, 5, 15, 30, 60, and 90 minutes' incubation at 37°C., tubes were removed and chilled promptly in an ice bath. Three-tenths ml. of homologous M protein extract was added to each tube and incubation was continued at 37°C. for an additional 15 minutes. The cultures were then chilled again in an ice bath and long chain counts were made from each tube as rapidly as possible.

It was evident that after brief exposure to penicillin, when the majority of streptococci were still viable, addition of homologous M protein resulted in prompt disintegration of the chains. As exposure to penicillin was continued there was progressive decrease in the ability of M protein to induce chain scission. After 90 minutes' exposure to penicillin at 37°C. when no viable or-

ganisms remained, addition of M protein extracts failed to decrease chain length (Fig. 5).

Factors Other than Anti-M Antibody Influencing the Chain-Splitting Mechanism

In the preceding experiments a metabolically active mechanism for splitting streptococcal chains was demonstrated after long chains were formed under the influence of anti-M antibody. The presence of this chain-splitting system in streptococci grown in the absence of anti-M antibody was now sought. The
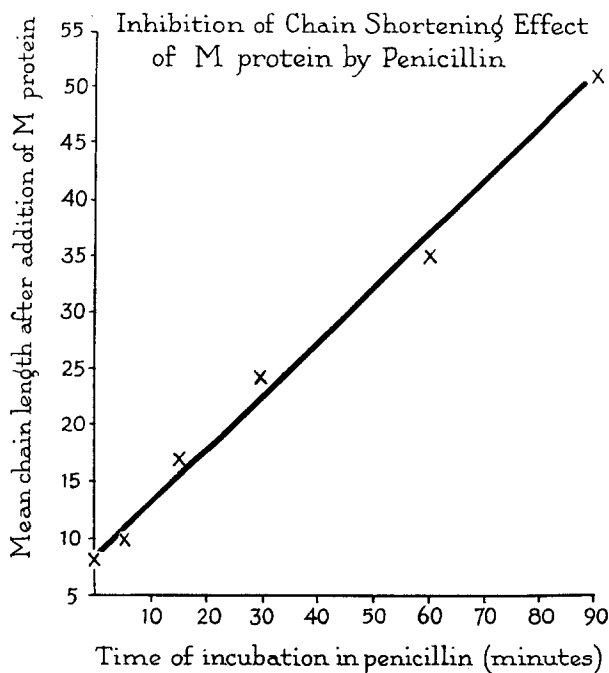

FIG. 5. Effect of penicillin on the chain shortening by homologous M protein extract. Long chains were exposed to penicillin for indicated times before M extract was added.

problem was approached by studying chain length of a large number of virulent and avirulent variants under different cultural conditions.

Relationship of Virulence to Chain Length.—It was observed consistently that all strains which resisted phagocytosis in human blood (free of homologous type antibody) produced a homogeneous population of relatively short chains when grown in serum-enriched Todd-Hewitt broth. These averaged approximately 5 to 15 cocci per chain. Very few individual chains of the population exceeded twice the average length.

When a spontaneous increase in chain length occurred upon frequent sub-culture, or after prolonged storage of stock cultures, it usually denoted dis-

sociation to a less virulent phase. This was indicated by diminished resistance of the strain to phagocytosis in human blood (demonstrated by bactericidal tests) and by a decrease in mouse virulence. Such changes often occurred before loss of M protein could be detected by semiquantitative capillary precipitin tests. In most instances repeated mouse passage rapidly restored the strain's former virulence and reduced its normal chain length.

Effect of Protein Enrichment of the Media.—Enrichment of Todd-Hewitt broth with normal rabbit serum resulted in a marked decrease in the length of the chains formed by virulent streptococci (Table II). This effect could be demonstrated by the addition of as little as 1 per cent serum to the broth medium. Normal rabbit, human, and sheep sera produced similar effects. Other sources of protein, such as crystalline ovalbumin and various human serum fractions, which were used as media enrichment had the same effect as whole serum.

Chain length of plain Todd-Hewitt broth cultures was also markedly affected by hydrogen ion concentration. At pH 7.5 to 8.0 the shortest chains were formed whereas below pH 7.0 or above 8.5 chains elongated strikingly. Subculture to serum-enriched broth at optimal pH resulted again in short chain growth.

Relationship of Synthesis of M Protein and Capsules to Chain Length.—When serum was added to Todd-Hewitt broth cultures of virulent Group A streptococci, greater amounts of M protein were synthesized and larger capsules were formed. The possibility was considered that the chain-shortening effect of normal serum in the media might be related to the synthesis of either or both of these surface components.

Variant strains of Group A streptococci were selected with respect to their ability to synthesize M protein and capsules. The chain length of these variants was determined after growth in Todd-Hewitt broth enriched with 10 per cent normal rabbit serum, in unenriched Todd-Hewitt broth, and in Todd-Hewitt broth containing 10 per cent homologous type rabbit antiserum.

Four groups of variants were studied; (a) M-positive encapsulated; (b) M-negative, encapsulated; (c) M-positive, non-encapsulated; and, (d) M-negative, non-encapsulated. The latter still reacted strongly with Group A antiserum.

Representative experiments are summarized in Table III. In broth enriched with normal serum only the encapsulated variants grew in very short chains, whether or not they contained M protein.

All variants grew longer in plain Todd-Hewitt broth than in serum-enriched broth but the increase in chain length was particularly evident in the encapsulated, M-negative variants. This was coincident with the marked decrease in capsule size observed when these strains were grown in plain broth unenriched with normal serum (Tables III and IV).

The striking specificity of the long chain effect of homologous antiserum for the M–anti-M system is demonstrated by the growth of these variants in their respective antisera. As expected, the M-positive, encapsulated strains showed striking increase in chain length. The M-negative, encapsulated variants did not

TABLE II

Effect of Serum Enrichment of Broth on Chain Length of Group A Streptococci

Strain	Type	Mean chain length in*		
		Plain Todd-Hewitt broth	Todd-Hewitt broth + 20 per cent normal rabbit serum	Todd-Hewitt broth + 20 per cent homologous rabbit antiserum
S1613	1	23	7	107
SS70	1	31	8	56
S628	3	39	19	100
34488	3	34	14	158
S1432	5	29	9	46
S43	6	44	12	112
S533	6	20	9	60
SF42	12	48	7	142
IH	14	22	8	71
44629	19	35	7	35
D24	30	27	9	139

* Each strain was cultured in the three media for 3 hours at 37°C. on the same day with identical inocula.

TABLE III

Relationship of Capsule and M Protein to Chain Length of Group A Streptococci

Strains	Mean chain length in		
	Normal serum broth	Plain Todd-Hewitt broth	Homologous antiserum broth
M^+ Caps.$^+$ (T-1 SS70)	6	25	66
M^- Caps.$^{+*}$ (T-14 S 23G)	14	68	14
M^+ Caps.$^{-\ddagger}$ (T-14 Latino)	39	41	90
M^- Caps.$^-$ (T-2 44/19)	45	87	92

* Three M-negative encapsulated strains were studied and all behaved similarly.

‡ Four unencapsulated M-positive strains were studied and all behaved similarly.

lengthen at all. Unencapsulated M-positive strains grew quite long in normal serum controls and, therefore, the increase in chain length due to antiserum was not very apparent. It was, however, definite. The M-negative unencapsulated variant designated T-2 44/19 in Table III also lengthened significantly in

antiserum. This was shown subsequently to be due to the synthesis of very small amounts of M protein by this strain when extracts of large numbers of these cells were made from serum-broth cultures. Such strains failed to yield detectable amounts of M protein by conventional extracts made from 40 ml. of Todd-Hewitt broth cultures.

The above results suggested that the chain-splitting system of streptococci was associated with the formation of capsules, the latter being enhanced by serum enrichment of the medium.

The Relationship of Capsule Formation to Chain Shortening.—

TABLE IV

Effect of Capsule Formation on Chain Length of Group A Streptococci

Normal serum in broth	Strains of streptococci					
	M-positive (T-12 WM)		M-negative (T-14 S23G)		M-negative (T-1 327W)	
	Capsule	MCL*	Capsule	MCL*	Capsule	MCL*
per cent						
0	++	56	+++	75	0	56
1	+++	32	++++	28	++	26
10	++++	11	+++++	13	+++	13
20	++++	10	+++++	14	+++	9
90	—‡	7	—‡	5	—‡	8

* MCL = mean chain length.

‡ India ink preparations not made because carbon particles agglutinated at high serum concentrations.

To further study the apparent relationship between capsule synthesis and chain scission, encapsulated strains were grown in Todd-Hewitt broth containing varying concentrations of normal rabbit serum. After 3 hour growth in these media, India ink preparations were made to demonstrate capsule formation and mean chain length was determined. Representative experiments are shown in Table IV.

With increasing concentrations of serum larger capsules were synthesized and chains grew shorter. At serum concentrations greater than 20 per cent capsules were difficult to see clearly because India ink particles tended to agglutinate. It was evident, however, that a general correlation existed between capsule formation and chain shortening. Although serum enrichment is also associated with increased production of M protein, it appeared, from the behavior of the M-negative strains, that the chain-splitting mechanism was independent of M protein itself.

*Effect of Hyaluronidase on Long Chain Formation.—*Although, in general, strains which formed the largest capsules also grew in the shortest chains, the

physical presence of the capsule itself did not appear to be essential for the chain-splitting system. Well encapsulated M^+ and M^- variants were grown in serum broth to which bull testis hyaluronidase[4] was added. Short chains were formed despite enzymatic removal of the capsule during growth (Table V). In the presence of hyaluronidase, however, homologous antiserum did not appear to produce as great an increase in chain length as that observed in control experiments made with the same antiserum to which hyaluronidase was not added (Table V). It was suspected that this partial inhibition of anti-M long chaining might be due to the loss of M protein from the cells which occurs when capsules are digested by hyaluronidase (16). That such was the case under the conditions employed seemed likely from the following experiments:

One liter of Todd-Hewitt broth culture was inoculated with 30 ml. of an overnight broth culture of an encapsulated Type 14 strain of streptococcus and was incubated for 3 hours at 37°C. India ink preparations showed large capsules which were two to three times the diameter of the cell. M protein could not be detected in the culture supernatants at this time when precipitin tests were made with standard absorbed typing antisera. The sedimented cells from these cultures were resuspended in 0.5 ml. Todd-Hewitt broth containing 300 turbidity-reducing units of hyaluronidase. After 30 minutes' incubation at 37°C. the cells were separated by centrifugation. India ink preparations now showed no visible capsules. The supernatant hyaluronidase digest was tested for M protein by capillary precipitin tests and showed a strong (3 plus) reaction. The cells, devoid of capsules, were extracted with acid and heat and the resulting extract also showed a strong (4 plus) precipitin test with homologous typing serum. The precipitin reactivity of both the hyaluronidase digest and the acid extract was destroyed by trypsin.

Therefore, the reduction in mean chain length of 3 hour cultures grown in homologous antiserum broth containing hyaluronidase might have been due to loss of some M protein present in the capsules of these young cultures. Not enough M protein was removed by this method to prevent some increase in chain length as compared to the normal serum hyaluronidase–broth controls (Table V).

Chain Formation and Disruption of Pneumococci.—Additional studies were made to determine whether long chain disruption of Group A streptococci by an excess of type-specific antigen was unique to this species or was a reflection of a more general phenomenon of coccal chain formation. A number of experiments were carried out employing *Diplococcus pneumoniae*, an organism which has been shown to form long chains in homologous antiserum (5).

Pneumococci behaved in a manner very similar to the streptococci. In homologous antiserum-broth the mean chain length of Types I, II, and III increased. Smooth, virulent strains grew in shorter chains than rough, avirulent strains in normal serum-broth and all strains grew longer in plain Todd-Hewitt broth. Addition of overnight culture supernates of Types I and II caused rapid

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[4] Infiltrase, Armour.

shortening of their homologous strains which had formed long chains in anti-serum-broth. The reactions were type-specific. Heat-killed long chains of pneumococci were insusceptible to disruption by homologous culture supernates containing specific soluble substance in excess of antibody. Heterologous anti-

TABLE V

Effect of Hyaluronidase on Chain Length of Group A Streptococci

Culture medium	Hyaluronidase	Capsule	Mean chain length*			
			M-positive strains		M-negative strains	
			T-1 SS70	T-14 IH	T-1 327W	T-14 S 23G
Normal serum-broth	Absent	+++	8	6	15	20
Normal serum-broth	Present	0	12	6	18	10
Antiserum-broth	Absent	++	92	62	11	36
Antiserum-broth	Present	0	23	22	9	30
Plain broth	Absent	+	22	17	71	90
Plain broth	Present	0	14	9	20	42

* Cultures incubated 3 hours at 37°C. and chains counted.

TABLE VI

Relationship of Growth Media and Capsules to Chain Length of Pneumococci

D. pneumoniae	Media	Mean chain length	Capsule
Virulent (SVI)	Normal serum-broth	4	+
	Plain Todd-Hewitt broth	15	+
	Homologous antiserum-broth	50	++++
Avirulent (D39R)	Normal serum-broth	9	±
	Plain Todd-Hewitt broth	30	±
	Homologous antiserum-broth	11	0

sera did not induce long chaining of the pneumococci after absorption, nor did heterologous culture supernates disrupt preformed long chains (Table VI).

DISCUSSION

The results reported strongly suggest that the scission of coccal chains occurs by an active enzymatic process which apparently is inhibited by the interaction of surface antigens with their specific antibodies. The chain-splitting system, which for convenience might be referred to as "scissin," appears to be most

active in encapsulated strains with or without M protein, and its synthesis seems to be influenced by the same cultural conditions that promote capsule and M protein formation.

Bisset (17) has called attention to the general relationship between formation of smooth colonies by virulent, encapsulated strains and shortchaining. Griffith (18) noted this relationship in studies on pneumococci. Howie (19) studied a Group B streptococcus ("Aronson's streptococcus") which mutated in the presence of acriflavin from smooth, encapsulated, mouse-virulent forms to rough, unencapsulated, avirulent variants. His photographs show the variants forming short chains and the R variants growing extremely long. It has been suggested recently (20) that an enzymatic mechanism is involved in the "unchaining" of strains of Streptococcus faecalis in which long chains were induced by growth in the presence of surface-active agents.

If the synthesis of a chain-splitting enzyme were under genetic control many of the reactions described here, and by others, might be explained, particularly if this hypothetical gene were closely linked to the gene controlling capsule synthesis. Loss of these closely linked genes by mutation would produce unencapsulated, long chaining variants. Strains containing the hypothetical gene would form large capsules and split into short units under proper nutritional and cultural conditions. Thus, factors producing either mutation or inhibition of enzyme action or synthesis would cause long chaining.

The long chaining effect of anti-M antibody is more difficult to account for hypothetically. The chain-splitting enzyme does not appear to be M protein itself because M-negative encapsulated variants shorten markedly in serum-enriched media. Activity of the enzyme does not depend upon the physical presence of the capsule since chains continue to grow short when the capsule is removed by hyaluronidase. Moreover, the effect of anti-M antibody is only apparent when M protein is present.

It is possible, therefore, that the union of antigen and antibody on the cell surface causes long chaining by interference with the action of the enzyme upon its cell wall substrate. The nature of the substrate is not yet apparent. Recently, Maxted (21) demonstrated a lysin of streptococcal cells which is produced by Group C streptococci infected with a streptococcal bacteriophage. This lysin also has been shown by Krause, Freimer, and McCarty (22, 23) to strip streptococci of their cell walls, leaving viable L forms. The lysin does not appear to destroy the serologically active components of the cell wall. The substrate of this lysin is as yet undefined. In view of the fact that the scissin system and the phage-associated lysin both apparently lack proteolytic activity there may be a relationship between the two.

The inhibition of chain scission of M-positive strains by anti-M antibody, and its reversal by M protein, is an exquisitely sensitive system for detecting minute quantities of either antigen or antibody, as demonstrated in the following report (24). Aside from the theoretical implications of this system, its

practical application to the study of streptococcal virulence and immunity is apparent.

SUMMARY

Group A streptococci which grew in long chains in the presence of homologous anti-M antibody were split into their original length by the addition of an excess of homologous M protein to the culture. The chain-splitting reaction showed temperature and pH optima (37°C., 7.5) and was completely inhibited at 0°C. or by heat-killing the long chains at 56°C. prior to the addition of M protein. Addition of sublethal doses of $HgCl_2$, or of penicillin, inhibited the chain-splitting reaction. Pneumococci behaved in entirely comparable fashion to streptococci in similar experiments.

Virulent strains of streptococci formed the shortest chains when broth media was enriched with serum. The chain-shortening effect of serum enrichment of the media was most apparent with encapsulated strains and under cultural conditions that favored capsule formation. Loss of capsules by mutation or by unfavorable growth conditions resulted in increase in chain length. The activity of the chain-splitting mechanism seemed to be independent of M protein, however, since encapsulated M-negative variants also formed very short chain in serum-enriched media. The physical presence of the capsule was not essential for chain shortening since enzymatic removal of the capsule with hyaluronidase during growth did not affect chain length.

These results strongly suggest that chain-splitting of streptococci and pneumococci occurs by an active metabolic mechanism, presumably enzymatic, which is inhibited by the union of surface antigens with specific antibody.

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