STUDIES OF PHAGOCYTOSIS OF GROUP A STREPTOCOCCI BY POLYMORPHONUCLEAR LEUCOCYTES IN VITRO*

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When Group A streptococci are mixed with human blood under appropriate conditions *in vitro*, the bacteria are rapidly engulfed and killed by leucocytes. Among the conditions (1) required for this reaction are: (*a*) sufficiently large numbers of leucocytes, (*b*) complement and perhaps other serum or plasma factors, (*c*) an efficient mixing system to ensure adequate opportunity for contact between the white cells and streptococci, and (*d*) type-specific antibody, which is necessary for phagocytosis only in the case of streptococcal strains containing M protein.

Efforts to develop an opsonophagocytic test for Group A streptococci, employing rabbit or mouse blood, have been unsuccessful. The lack of bactericidal action in blood of these animals has been attributed by some to failure of the leucocytes to kill engulfed microorganisms (2, 3), while others have implicated deficiencies in plasma factors and inadequate phagocytosis (1, 4). Furthermore, recent studies have demonstrated that the differences between rabbit and human blood are not entirely consistent in this regard; blood from certain apparently healthy human beings is not satisfactory for use in the bactericidal test on streptococci, while on rare occasions a rabbit is found whose blood serves quite well for this purpose (5).

This communication presents studies on interactions between polymorphonuclear leucocytes and Group A streptococci *in vitro*. The results indicate that under certain conditions an anti-phagocytic effect is exerted by the streptococcal hyaluronic acid capsule as well as by M protein. All human plasma or serum specimens thus far studied contain a factor, apparently neither enzyme nor antibody in nature, which counteracts the phagocytosis-resisting action of the capsule. In contrast, rabbit serum appears to be devoid of this factor.

Methods

Peritoneal exudates were collected from adult New Zealand Red rabbits by a technique previously described (6), with slight modifications. The essential features of this procedure were as follows. Three hundred ml. of sterile isotonic saline containing 1 mg. per ml. glycogen

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(Amend Drug and Chemical Co. Inc., New York) were injected intraperitoneally. Three to 4 hours later, a No. 15 perforated needle was inserted into the peritoneal cavity with the rabbit in the prone position. The exudate was allowed to flow by gravity into a flask containing 10 mg. of heparin. Ordinarily the 100 to 200 ml. of exudate recovered contained approximately 5×10^6 leucocytes per $mm.^3$, of which over 95 per cent were polymorphonuclear ones. Cells were separated from the exudate supernatant fluid by spinning (International centrifuge, size 2) at 1000 R.P.M. for 10 minutes. All operations were performed at room temperature.

Streptococcal strains were kindly provided by Dr. Rebecca Lancefield. These were maintained in the frozen state in small aliquots until time of use, and were then cultured in Todd-Hewitt broth to which was added a sterile solution of bovine albumin (fraction V) to a final concentration of 0.3 per cent. Young subcultures were made by inoculating 5 ml. of Todd-Hewitt albumin broth with 0.1 ml. of an 18 hour old culture. These subcultures were then used after 3 hours growth at 38°C. Todd-Hewitt broth containing 10 per cent human serum, 10 per cent rabbit serum, or 1 per cent defibrinated rabbit blood was also used for culturing the organisms in some experiments.

Bactericidal tests were done in 10×75 mm. pyrex text tubes. After the white cells, diluting fluids, and other test ingredients had been placed in these tubes to give the desired cell count in a volume of 0.9 ml., 0.1 ml. of approximately a 1:3000 dilution of the streptococcal culture was added. Streptococci were diluted in the suspending medium used for the bactericidal test; initially this was the supernatant fluid from the rabbit peritoneal exudate, while later Gey's balanced salt solution containing 10 per cent rabbit serum was employed. The tubes were sealed with sterile rubber stoppers. Promptly after initial mixing an aliquot was removed for plate count of the numbers of streptococcal units in the test system. The tubes were then rotated end-over-end at 8 R.P.M. at 38°C. At various time intervals aliquots were removed. Pour plates (proteose agar containing 2 per cent defibrinated rabbit blood) were made with measured volumes of the white cell——streptococcal suspensions and of tenfold serial dilutions of these in Todd-Hewitt broth. After overnight incubation at 38°C., colony counts permitted calculation of the number of surviving streptococcal units per milliliter of original suspension.

Antisera prepared in rabbits against certain streptococcal strains were kindly provided by Dr. Rebecca Lancefield.

Observations were made on encapsulation of streptococci and on their phagocytosis in the leucocyte mixtures as follows. A loopful of the suspension to be studied was mixed on a clean slide with a loopful of a 1:5 dilution in saline of a finely divided, well dispersed carbon suspension (Pelikan, Gunther-Wagner, Hannover, Germany). A coverslip was applied and the mixture examined under high power ($\times$ 450), utilizing phase contrast optics. Capsules were readily detected by this procedure and estimation could also be made of the number and length of extracellular streptococcal chains.

Bovine testicular hyaluronidase (Worthington Biochemical Corp., Freehold, New Jersey) was dissolved at 5 mg. per ml. in sterile isotonic saline and stored at 4°C. Appropriate quantities of this stock solution were added at the time of bactericidal testing. A fresh solution of hyaluronidase was made each week to minimize the effects of variable deterioration of activity in the stock solution.

Blood obtained from normal rabbits and healthy human beings was rimmed after clotting, incubated at 38°C. for 1 hour, and centrifuged. The serum, distributed in small aliquots and stored at −20°C., was used within 1 month of the time of collection.

Human leucocytes were obtained from blood by the dextran sedimentation technique. Forty ml. of blood was drawn into a syringe previously moistened with 0.4 ml. of a 10 mg. per ml. solution of heparin. After addition of 2 ml. of 12 per cent dextran in water (Abbott

Laboratories, Chicago) and mixing, sedimentation was allowed to proceed in 15 × 150 mm. test tubes for 2 hours at room temperature. The supernatant cell-rich plasma layer was then removed and spun at 500 R.P.M. (International Centrifuge, size 2) for 10 minutes, bringing down cells but leaving most of the platelets in suspension. The cell button was washed twice with isotonic saline in the centrifuge and then suspended in 10 ml. of saline for total and wet differential counts.

RESULTS

The Fate in Vitro in Rabbit Peritoneal Exudates of Streptococci from Young and Old Cultures

The early experiments were performed by adding streptococci to rabbit peritoneal exudates and observing the fate of the bacteria on mixing *in vitro*.

TABLE I

Fate of Various Group A Streptococci on Mixing in Vitro with Rabbit Peritoneal Exudates

Type and strain of Group A streptococcus	Hyaluronidase production	M protein	Capsule formation in young cultures	Killing in rabbit phagocytic system of	
				Young (3 hr.) cultures	Old (18 hr.) cultures
Type 3, D58x (R^+)	0	0	+	0	+
" , B930/24 (R^-)	0	+	+	0	+
" , D121 ($R^\pm$)	0	+	+	0	+
Type 4, C748	+	0	0	+	+
" , C155/5	0	0	0	+	+
Type 22, B401	+	0	0	+	+
" , T22/117	+	0	0	+	+
Type 6, S43MA	0	0	+	0	+
" , S43FL	0	+	+	0	+
" , S43G	0	0	0	+	+
" , B410	0	+	0	+	+

The concentration of granulocytes in each experiment was adjusted to 25,000 per mm.^{3} (over 95 per cent polymorphonuclears) by centrifuging the cells and resuspending them in an appropriate volume of the supernatant fluid. Initially 18 hour old cultures of various streptococcal strains were used; all strains so studied were killed on mixing with rabbit peritoneal exudates. However, 3 hour old subcultures of several of these strains multiplied in the same test system. Results of these experiments do not warrant detailed presentation, but are summarized in Table I. It is seen that there was good correlation between resistance to killing and the presence of a capsule.

In these experiments, as well as in those to be discussed in later sections of this report, specimens were frequently included in which tubes were held stationary instead of being tumbled, and additional controls were agitated but

white cells were omitted. In every instance streptococci grew in these controls, indicating that bactericidal activity, when observed, was due to phagocytosis and intracellular killing.

The results shown in Table I suggested, but by no means established, that hyaluronic acid capsules rendered Group A streptococci relatively resistant to engulfment by rabbit polymorphonuclear leucocytes. Obviously young cultures might differ from old ones in many ways in addition to encapsulation. Further experiments were done in which a single streptococcal culture was tested for susceptibility to phagocytosis at 30 minute intervals throughout its growth; under the conditions used, resistance to killing in the rabbit exudate system appeared at about hour 1 and disappeared at about hour 6 of the growth cycle. Microscopic observations revealed that this coincided precisely with the period during which capsules were visible.

Subcultures of streptococci in broth containing a final concentration of 100 μg. hyaluronidase per ml. of medium remained unencapsulated throughout their growth cycle. Three hour old subcultures in hyaluronidase-broth manifested resistance to phagocytosis and killing similar to that of the young cultures grown in ordinary Todd-Hewitt broth. This at first suggested that hyaluronic acid capsule was not the important anti-phagocytic factor of the young cultures. However, microscopic studies revealed that the 3 hour old subcultures grown in the presence of hyaluronidase, although devoid of capsule at the time of addition to the rabbit exudate test system, rapidly became fully encapsulated after transfer to this system, large capsules being visible within 10 minutes of transfer. This finding indicated that hyaluronidase activity in the final test system was necessary for reliable appraisal of the relationship between encapsulation and resistance to phagocytosis.

The Effects of Hyaluronidase and of Antiserum on Susceptibility of Various Strains of Streptococci to Phagocytosis by Suspensions of Rabbit Granulocytes

The supernatant exudate fluid varied considerably from time to time in its ability to antagonize hyaluronidase activity. Therefore the suspending medium for opsonophagocytic tests was changed to a balanced salt solution (Gey's) containing 10 per cent fresh serum. It was noted that serum concentrations lower than 10 per cent did not regularly allow phagocytosis at maximal rate, presumably because of the requirement for complement in this process. On the other hand, systems containing more than 20 per cent serum manifested variable inhibition of hyaluronidase at the concentration of this enzyme (100 μg. per ml.) employed in the test. All experiments discussed in this section were done on 3 hour old subcultures of streptococci. Washed rabbit peritoneal exudate granulocytes were present at 25,000 per mm.^{3}. Additions made to this test system in each experiment included homologous antiserum, heterologous antiserum, and hyaluronidase in various combinations.

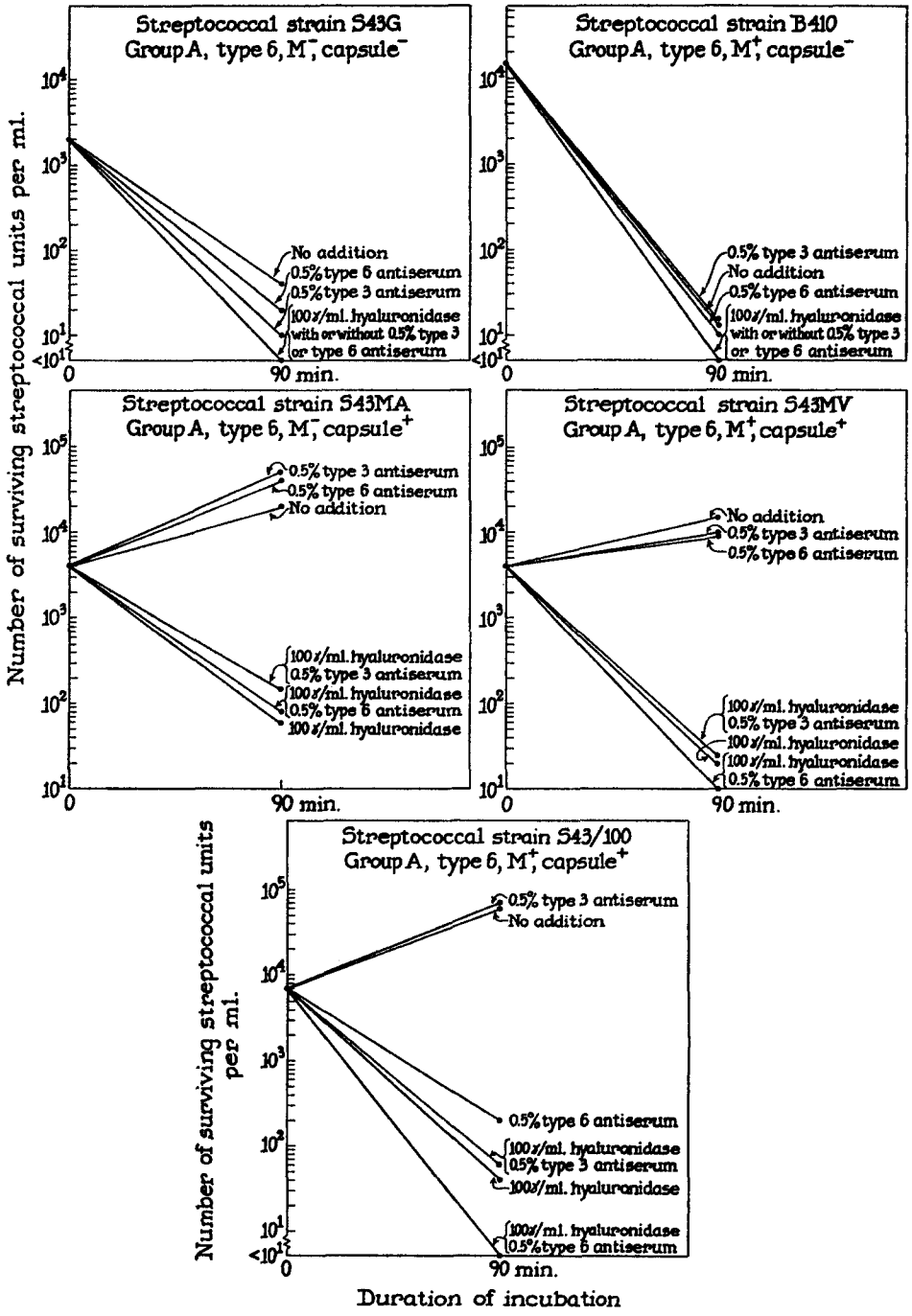

FIG. 1. The influence of hyaluronidase and of antiserum on the fate of various type 6 streptococci mixed *in vitro* with suspensions of rabbit polymorphonuclear leucocytes.

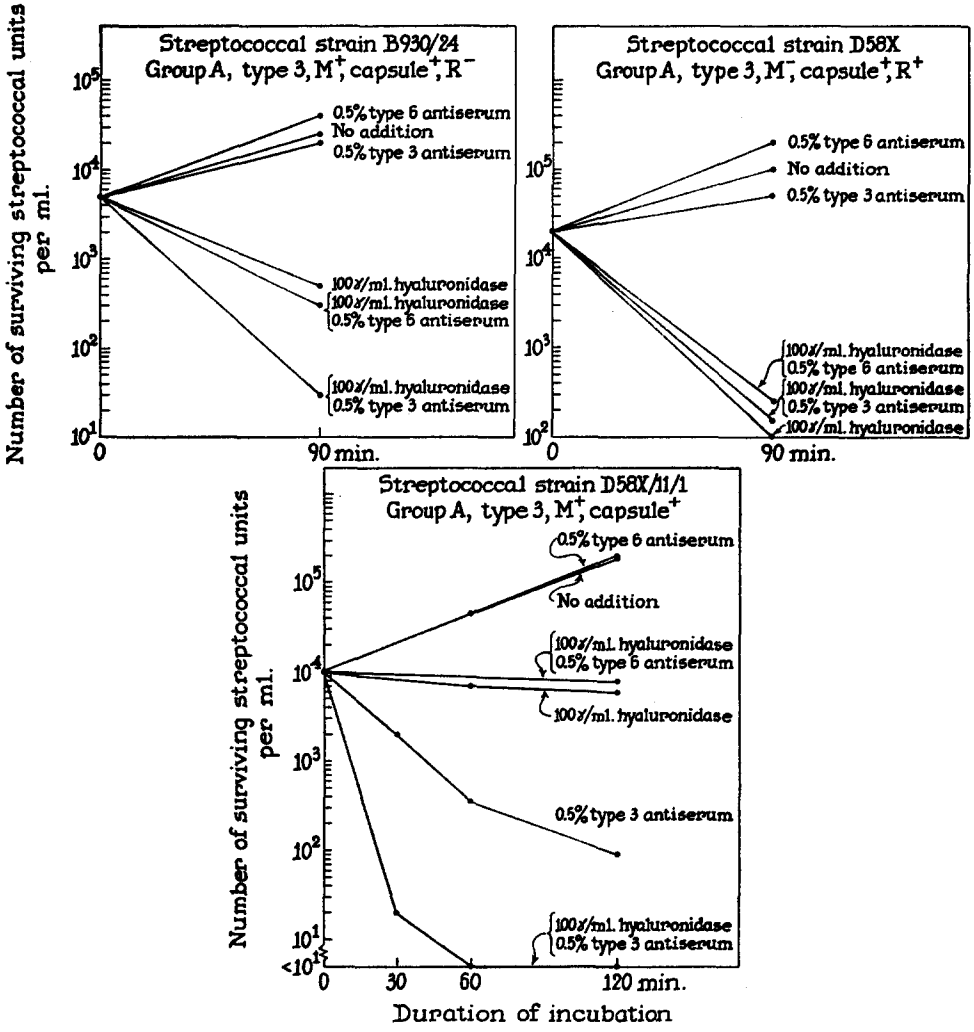

FIG. 2. The influence of hyaluronidase and of antiserum on the fate of various type 3 streptococci mixed *in vitro* with suspensions of rabbit polymorphonuclear leucocytes.

Fig. 1 shows the results of phagocytosis-bactericidal tests on several strains of type 6 streptococci. The non-capsulated strains, B410[1](M+) and S43G (M−), were both killed without addition of antiserum or hyaluronidase. In contrast, the encapsulated strains S43MV[1](M+) and S43MA (M−) multiplied in the basal test system and in those specimens containing type 3 or type 6 antiserum,

[1] Strain B410 contained only small amounts of M protein detectable in precipitin tests, whereas strains S43MV and S43/100 yielded extracts with large and approximately equal quantities of M substance. Only S43/100 among these strains was highly virulent for mice.

while addition of hyaluronidase, with or without either antiserum, led to killing. The encapsulated strain S43/100[1](M+) exhibited somewhat different behavior, growing in the unmodified test system and in the presence of added heterologous antiserum, while addition of homologous antiserum resulted in moderate bactericidal action. Hyaluronidase alone or in combination with type 3 antiserum also led to moderate killing of S43/100. The combined addition of hyaluronidase and type 6 antiserum resulted in most efficient elimination of this strain.

Results of similar studies on type 3 strains of Group A streptococci are shown in Fig. 2. These were done to confirm the findings obtained with the type 6 strains, and also to investigate the possible role of R antigen in this phagocytic system. Strain D58X (M−, R+, encapsulated) multiplied in the presence of either type 3 or 6 antiserum, while the numbers of surviving streptococcal units were markedly reduced when hyaluronidase or combinations of hyaluronidase and either antiserum were added. Strain B930/24[2](M+, R−, encapsulated) also grew in the control test system with no additions, and this growth was not influenced by addition of homologous or heterologous antiserum. Hyaluronidase alone or hyaluronidase plus type 6 antiserum led to a moderate lethal effect, while addition of both hyaluronidase and type 3 antiserum resulted in marked killing. Strain D58X/11/1[2](M+, encapsulated) increased in numbers when no additions were made to the system and also when heterologous antiserum was added, while homologous antiserum brought about moderate killing. The numbers of viable streptococcal units remained relatively constant in the presence of hyaluronidase with or without type 6 antiserum. Addition of hyaluronidase and type 3 antiserum to the test system led to rapid and complete elimination of this strain. In the figure presenting results with strain D58X/11/1, enumeration of bacteria is recorded at frequent intervals during the experiment, rather than only at the outset and at 90 minutes. The curves so obtained show the rapidity with which phagocytosis and killing can be accomplished provided conditions are optimal for these processes.

Several other observations, not shown in the figures, were made in the course of these experiments. Hyaluronidase solutions heated at 100°C. for 10 minutes lost completely their capacity to promote phagocytosis of streptococci. Addition of other basic proteins, such as lysozyme and ribonuclease, did not lead to engulfment of encapsulated streptococci by rabbit leucocytes. Streptococcal subcultures in Todd-Hewitt broth containing 10 per cent human, rabbit or bovine serum, or 1 per cent rabbit blood behaved the same as those grown in Todd-Hewitt albumin broth.

[2] Both strains B930/24 and D58X/11/1 contained large amounts of M protein as measured in precipitin tests, and both were virulent for mice.

Comparison of Human and Rabbit Leucocytes and Serum in Their Capacity to Promote Phagocytosis and Killing of Group A Streptococci

It thus seemed clear that encapsulated streptococci resisted phagocytosis in the rabbit leucocyte–rabbit serum system. Further studies were required to determine whether or not this phenomenon bore any relation to the known difference between rabbit and human blood in their suitability for opsono-phagocytic tests for detection of type-specific streptococcal antibodies. In these experiments an encapsulated, M^- streptococcal strain, S43MA, was employed so as to permit evaluation of the anti-phagocytic role of the capsule uncom-

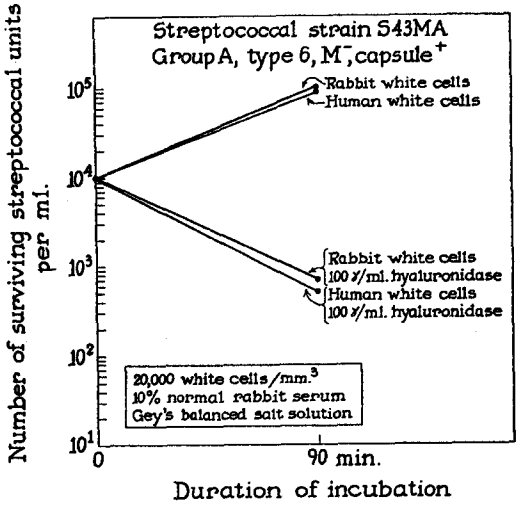

FIG. 3. Comparison of human and rabbit leucocytes in their capacity to destroy encapsulated streptococci *in vitro* in a medium containing rabbit serum.

plicated by additional similar effects of M protein. Young subcultures of this strain were added to suspensions of human or rabbit white cells in Gey's solution containing human or rabbit serum, and fate of the bacteria was determined on mixing at 38°C. Human leucocytes were prepared from blood by the dextran sedimentation technique (see Methods).

The results shown in Fig. 3 indicate that rabbit and human white cells did not differ in their capacity to engulf and destroy streptococci. In a medium containing 10 per cent rabbit serum, neither human nor rabbit leucocytes were able to kill the test microbes efficiently unless hyaluronidase was added to the system. In the presence of hyaluronidase, both cell types handled the streptococci in essentially identical fashion. No clumping or other detectable damage to human leucocytes was evident in the presence of rabbit serum.

Fig. 4 presents results of a similar experiment in which 10 per cent human

serum in Gey's solution was employed as the suspending medium. Meaningful results could not be obtained using rabbit white cells in this test system, since they were promptly agglutinated when suspended in a medium containing human serum. Several human sera from each of the ABO blood groups were used, but each produced extensive damage to rabbit white cells as evidenced by clumping and permeability to trypan blue. Human leucocytes were not clumped or otherwise detectably damaged, and, as is shown in Fig. 4, accomplished slight killing of the streptococci. The lethal action was considerably enhanced

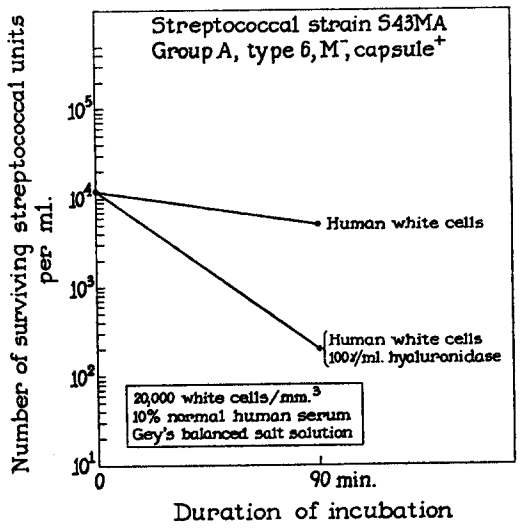

FIG. 4. The influence of hyaluronidase on the phagocytosis *in vitro* of encapsulated streptococci by human leucocytes in a system containing 10 per cent human serum.

by addition of hyaluronidase, showing that in this particular human cell–human serum system, streptococcal capsules exerted an anti-phagocytic effect. The slight killing of streptococci by human leucocytes in the 10 per cent human serum medium suggested that a capsule-neutralizing factor might be present in limited amounts in human serum, and that higher serum concentrations might be required for its unequivocal demonstration.

Further experiments were therefore done employing human leucocytes and various concentrations of fresh serum from the same person. These were carried out in a medium containing 10 per cent fresh rabbit serum to provide adequate complement. The results of a typical study are shown in Fig. 5. Clearly demonstrated is the role of human serum in promoting phagocytosis of this encapsulated, M^- streptococcus. At concentrations of human serum lower than 10 per cent, the bacteria were not engulfed and hence multiplied. Ten per cent

human serum in the system resulted in an essentially steady streptococcal population, with the rates of phagocytosis and extracellular growth approximately balancing each other. At concentrations of human serum of 30 per cent or greater, rapid phagocytosis and killing of the microbes was seen, the degree of this phagocytosis-promoting action being, in fact, even more striking than that resulting from addition of hyaluronidase to the medium.

In contrast, addition of rabbit serum in concentrations as high as 90 per cent failed to promote phagocytosis of the streptococci.

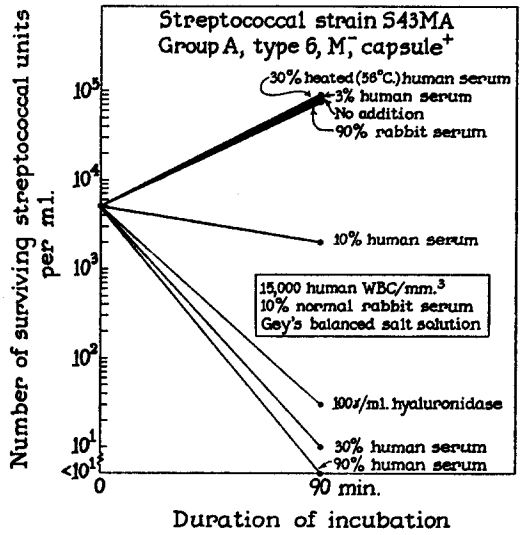

FIG. 5. Demonstration that a factor promoting phagocytosis *in vitro* of encapsulated streptococci by human leucocytes is present in fresh human serum, but absent in rabbit serum and in heated human serum.

Only a few properties have been determined for the human serum factor which promoted phagocytosis of encapsulated streptococci. As is shown in Fig. 5, heating at 56°C. for 30 minutes destroyed the activity. Heparinized plasma served as well as serum in the phagocytosis tests while pooled human gamma globulin (Lederle) was without effect. No differences were found in the course of encapsulation of streptococci incubated in rabbit and human sera which were, respectively, ineffective and effective in rendering encapsulated streptococci susceptible to phagocytosis. Human serum did not produce detectable swelling or lysis of the capsules.

There may be a significant difference among individuals in the amount of the capsule-neutralizing factor contained in their serum. With the 3 human sera thus far studied, the concentration required for opsonization of encap-

sulated M^- streptococci was less than 5 per cent, 10 to 20 per cent, and 40 per cent, respectively. Ten rabbit sera have been investigated, and none contained measurable amounts of this phagocytosis-promoting factor.

DISCUSSION

Many previous investigators have inquired into the differences between human blood and that of rabbits or mice in relation to their capacity to kill Group A streptococci *in vitro* (1–5). Most observations show that, in the presence of homologous type antibody, virulent, M^+ streptococci are killed on mixing with fresh human blood, but survive or multiply in mouse or rabbit blood; similarly avirulent M^- strains are destroyed by human blood even in the absence of antibody, whereas they often grow in animal blood. The notion that this behavior might reflect differences in the ability of leucocytes from various species to kill ingested streptococci (2, 3) seems to have been disproved by studies of Rothbard (1) and Wilson *et al.* (7), as well as by the results presented in this communication. Previous investigations have also excluded differences in numbers of leucocytes as the explanation for variation in streptococcicidal activity of blood from various animals (3, 4). Furthermore, rates of proliferation of streptococci in serum or heated blood from man and animals are not significantly different (4). Thus, as concluded recently by Wiley (4), the rate of engulfment of streptococci by leucocytes in human blood must be more rapid than that in mouse or rabbit blood. Our investigation supports this conclusion, and also confirms and extends the earlier studies of Rothbard (1), in which the rapid phagocytosis of streptococci in human blood was attributed to a serum factor of unknown nature. The results presented here indicate moreover the probable reason for accelerated engulfment of streptococci in human blood as compared to that in rabbit blood. Human serum contains a factor which counteracts the anti-phagocytic effect of streptococcal hyaluronic acid capsules, whereas rabbit serum lacks this factor.

The anti-phagocytic activity of streptococcal hyaluronic acid capsules has been demonstrated previously (8–11). Evidence obtained in the present studies to support this concept is of two types. Firstly, there was correlation between presence of capsules among various strains or during the growth cycle of a given strain and resistance to engulfment by rabbit white cells *in vitro*. Secondly, the use of hyaluronidase under conditions appropriate for its enzymatic action resulted in markedly increased susceptibility to phagocytosis. Heated solutions of hyaluronidase had no effect on phagocytosis, nor did solutions of other basic proteins such as lysozyme or ribonuclease, suggesting that hyaluronidase acted in the system by virtue of its ability to strip off the capsule, and not by simple combination with the surface and non-specific opsonization.

The influence of type-specific antibody on phagocytosis of M^+ streptococci in the rabbit leucocyte–rabbit serum system was variable; addition of type-

specific antibody led to engulfment and moderate killing of some strains (D58X/11/1 and S43/100), whereas other M^+ strains (B410, S43MV, and B930/24) and all M^- encapsulated strains resisted phagocytosis even in the presence of antibody. This behavior might be dependent on the quantity and/or location of M protein in the bacterial cells. If some strains contain large amounts of M protein located at or near the surface, combination of antibody with this antigen might result in effective opsonization and masking of the hyaluronic acid capsule. On the other hand, strains with little or no M protein accessible at the surface might well combine with insufficient antibody to coat the surface effectively, in which event the capsule would continue to exert its anti-phagocytic effect.

One technical aspect necessary for success of these studies was the choice of a suitable test medium. Hyaluronidase added to broth cultures of streptococci prevented the appearance of visible capsule, but organisms so grown very rapidly became fully encapsulated on transfer to a blood or exudate medium. Effective elimination of streptococcal capsules during phagocytic tests required hyaluronidase in an active form in the final test system. Serum concentration of the medium thus became critical; too much serum antagonized hyaluronidase, while too little provided inadequate complement.

A role for the hyaluronic acid capsule in influencing susceptibility of streptococci to phagocytosis *in vitro* in no way conflicts with or detracts from the well established function of M protein in this regard (1, 11, 12). It is apparent that some streptococci possess two surface components, M protein and hyaluronic acid, either or both of which are able to protect the bacteria from engulfment by leucocytes under certain conditions. The recent studies of Foley and Wood (11) show that both M protein and hyaluronic acid capsules also exert anti-phagocytic effects when tested under surface phagocytosis conditions.

The nature of the factor in human serum which counteracts the anti-phagocytic effects of streptococcal capsules remains uncertain. The lack of activity of pooled concentrated human gamma globulin, and the heat lability of the serum factor make it unlikely that an antibody to hyaluronic acid is at work. Streptococci develop capsules well in human serum, so that no enzymatic action similar to that of hyaluronidase is present to explain the phenomenon. It is tempting in view of the heat lability to attribute to complement the opsonizing action of human serum on encapsulated streptococci. Quantitative differences between human and rabbit serum in their complement content, at least as measured in hemolysis tests, is not great; qualitative differences in complement from various animal species have been demonstrated, but no information is available on their importance in the phagocytic process.

The number of human sera thus far studied is small, and no statements can be made about relationship of the streptococcal capsule-neutralizing factor to

age, genetic background, or history of previous streptococcal or other infections.

SUMMARY

Studies have been made on phagocytosis and killing of Group A streptococci during mixing with suspensions of leucocytes *in vitro*. Under appropriate test conditions an anti-phagocytic effect can be demonstrated for the streptococcal hyaluronic acid capsule as well as for its M protein.

The results obtained suggest an explanation for the suitability of human, but not rabbit, blood for opsonophagocytic tests designed to measure type-specific streptococcal antibodies. Human sera contain a factor which counteracts the anti-phagocytic effects of streptococcal hyaluronic acid capsules, and hence human blood serves well for detection of antibodies which combine with the only other phagocytosis-resisting component of this microorganism, namely M protein. In contrast, rabbit sera contain none of this factor, and addition of antibody to M protein to phagocytic test systems employing rabbit serum does not necessarily render the streptococci susceptible to engulfment by white cells, since the hyaluronic acid capsule may continue to interfere with phagocytosis.

The nature of the human serum factor which opsonizes encapsulated streptococci is unknown. It does not appear to be an antibody or an enzyme capable of depolymerizing hyaluronic acid.

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