PROTECTIVE EFFECT OF HYALURONIDASE AND TYPE-SPECIFIC ANTI-M SERUM ON EXPERIMENTAL GROUP A STREPTOCOCCUS INFECTIONS IN MICE

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PLATES 13 TO 15

(Received for publication, June 17, 1948)

The pathogenicity and invasiveness of group A streptococci for man, as well as for laboratory animals, have been shown to be closely correlated with the presence of the M protein; conflicting evidence, however, has been obtained by several groups of investigators with regard to the rôle of the hyaluronic acid capsule in the manifestation of virulence of these microorganisms.

A number of studies have been carried out in an effort to ascertain the effect of these substances on the pathogenesis of streptococcal infections. Several workers have studied the protective action in mice, infected with group A streptococci, of immune serum containing antibodies directed against the M substance. Others have studied the protective effect of hydrolyzing the capsular material with an enzyme, hyaluronidase. As a result of the antibody studies the M substance has been shown to be essential for the exhibition of virulence, since anti-M antibodies provide active and passive protection of mice against infection with virulent streptococci of homologous type (1–3). On the other hand, the relation of the hyaluronic acid capsule to mouse virulence is not so well defined. While Hirst (4) was able to protect mice infected with encapsulated group C streptococci by treatment with leech extract containing hyaluronidase, he was not able to protect mice infected with group A streptococci; although the capsular materials of both these groups of streptococci have been shown by Seastone (5) to be chemically similar. Blundell (6) obtained no definite protection against group A streptococcus infections in mice with crude bovine testicular hyaluronidase but observed a greater mean survival time; and McClean (7), using enzyme from a similar source, failed to obtain protection in mice infected with either group A or group C streptococci.

However, Kass and Seastone (8), who recently succeeded in protecting mice to some extent against infection with group A streptococci by using bovine testicular hyaluronidase, ascribed the failure of earlier workers to the use of insufficient amounts of enzyme and to too long an interval between injections. Both of these factors tended to permit the streptococci to regenerate their capsules in the infected host.

In view of these conflicting reports as to the protective effect of hyaluronidase, it seemed desirable to reinvestigate this problem and to attempt to determine the relative significance of the hyaluronic acid capsule and the M protein in the virulence of group A streptococci.

For this purpose, 5 strains of group A streptococci were selected, each of which was available in a glossy and matt form. The glossy variants were mouse-avirulent and produced little or no M protein; the matt variants were made mouse-virulent by animal passage and elaborated large amounts of the M substance. Both variants of each of the 5 strains were morphologically well encapsulated.

As criteria of virulence, two different and distinct systems were employed: (1) an *in vitro* test involving the capacity of the streptococcal cells to resist phagocytosis by leukocytes in human blood; and (2) an *in vivo* test involving the ability of group A streptococci to kill mice following intraperitoneal inoculation. The protective effect of anti-M serum and an enzyme, hyaluronidase, which hydrolyzes the capsular material, was also studied in the *in vivo* system against group A streptococcal infections in mice.

Materials and Methods

Strains of Hemolytic Streptococci.—The 5 strains of group A streptococci were selected because each represented a different serological type, each had a glossy and a matt variant, and both variants were capable of producing good capsules under appropriate conditions.

Griffith's (9) original type strains were used for types 1, 3, and 19. They were designated by him as strains SF 130/2, Lewis opaque 47T, and SF 73/4 and catalogued in this laboratory as T1, T3, and T19 respectively. Strain NY5 was isolated by Stevens and Dochez (10) and used by Griffith as the representative type 10, now designated type 12. Strain S23, type 14, was isolated from the throat of a patient with lobar pneumonia (11). The group C strain, designated D181 in this laboratory, was isolated by Seastone (5) from a guinea pig with chronic lymphadenitis and referred to as strain 4.

Media for Preparation of Cultures.—Todd-Hewitt filtered broth was the most favorable of several media tried for production of well formed capsules (2). Well encapsulated streptococci in the logarithmic phase of growth were prepared by adding to 9 parts of this medium one part of an actively growing 12 hour culture and incubating in a water bath at 37°C. for 3 to 4 hours. Large capsules (Figs. 1–10) were demonstrable by the moist India ink method (12) in both variants of all the strains studied. The cultures were standardized turbidimetrically at a wave length of 520 mμ before each experiment, and this was checked by pouring rabbit blood agar plates to estimate the bacterial population.

The methods of preparing the antisera and M extracts (13, 14), as well as the techniques used in the precipitin and bacteriostatic tests (15, 16), have previously been described. The method for preparing crude bovine testicular extract containing hyaluronidase was that of Kass and Seastone (8). For the mouse virulence and protection experiments, mice of the Rockefeller Institute strain, weighing 15 to 20 gm., were employed.

EXPERIMENTAL

The Relationship between the Production of M Protein and Mouse Virulence of Group A Streptococci.—Experiments were devised to determine the comparative capacities of the equally well encapsulated glossy and matt variants to produce the M substance and to kill mice. From 45 cc. of actively growing 3 hour broth cultures of the encapsulated variants, after a sample was removed for the mouse virulence tests, crude M extracts were prepared. Twofold serial dilutions of these extracts were tested by the capillary precipitin method (15) with homologous, absorbed anti-M serum, as recorded in Table I. A

distinct difference in the amount of the M substance elaborated by the glossy and matt variants of each pair of strains is apparent. Dilutions as high as 1:32 of the extracts of the matt variants showed positive reactions, whereas the undiluted extracts of the glossy variants gave only weak or no precipitin reactions.

For the mouse virulence tests, tenfold serial dilutions were prepared from samples removed from the cultures tested for M substance. The results of these tests are summarized in Table II. A marked difference in mouse virulence between the glossy and matt strains was found in spite of the fact

TABLE I

Production of Type-Specific M Antigen by Encapsulated Variants of Group A Streptococci Precipitin Reactions with M Extracts and Homologous Antisera

Variant	Glossy					Matt				
Strain	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type	1	3	12	14	19	1	3	12	14	19
Titration of M extract										
1:1	±	±	±	0	±	+++	+++±	+++	+++	+++
1:2	0	0	±	0	0	+++	++	+++	+++	+++
1:4	0	0	0	0	0	++	++	+++	++±	+++
1:8	0	0	0	0	0	++	++	++±	++±	+++
1:16	0	0	0	0	0	++	+±	++±	++±	++
1:32	0	0	0	0	0	+	±	±	+	+
1:64	0	0	0	0	0	0	0	0	0	0

In titration of M antigen, degree of precipitation is indicated by a ++++ to ± scale; 0 represents no precipitation.

that each variant elaborated equally good hyaluronic acid capsules. All of the encapsulated glossy variants were relatively mouse-avirulent; the encapsulated matt variants were highly virulent, as indicated by death in 3 to 4 days occurring in mice infected with 10^{-8} cc. of culture, containing 2 to 3 microorganisms. Examination of selected mice that died all revealed encapsulated streptococci in the peritoneal exudate or heart blood. In additional experiments it was shown that polymorphonuclear neutrophils and monocytes in the exudate, obtained from the peritoneal cavity of mice inoculated with the encapsulated glossy variants, were actively phagocyting the streptococci, but only rarely were the encapsulated matt variants taken up by the phagocytic leukocytes.

While these experiments clearly demonstrate the relationship of the M protein to mouse virulence, they do not bring out any evidence that the hyaluronic acid capsule has an effect in enhancing the virulence of these

TABLE II

Mouse Virulence of Encapsulated Variants of Group A Streptococci

Variant	Glossy					Matt				
Strain	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type	1	3	12	14	19	1	3	12	14	19
Dose cc.										
10^{-1}	D1	D1	D1	D1	D1	D1	D1	D1	D1	D1
	D1	D1	D2	D1	D1	D1	D1	D1	D1	D1
	D2	D1	D2	D2	D1	D1	D1	D1	D1	D1
10^{-2}	S	D2	S	D1	D1	D1	D1	D1	D1	D1
	S	D2	S	D2	D1	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
10^{-3}	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D1
10^{-4}	S	S	S	S	S	D1	D1	D1	D1	D1
	S	S	S	S	S	D1	D1	D1	D1	D2
	S	S	S	S	S	D1	D1	D1	D1	D2
10^{-5}	S	S	S	S	S	D1	D1	D2	D1	D2
	S	S	S	S	S	D1	D1	D2	D2	D2
	S	S	S	S	S	D1	D1	D2	D2	D2
10^{-6}	S	S	S	S	S	D2	D2	D2	D2	D2
	S	S	S	S	S	D2	D2	D2	D2	D3
	S	S	S	S	S	D3	D2	D2	D2	D3
10^{-7}	S	S	S	S	S	D2	D2	D2	D2	D3
	S	S	S	S	S	D3	D2	D2	D3	D3
	S	S	S	S	S	D3	D3	D3	D2	S
10^{-8}	S	S	S	S	S	D3	D3	D3	D3	D3
	S	S	S	S	S	D3	D4	D3	D3	D3
	S	S	S	S	S	D3	D4	D3	S	S

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

streptococci for mice. Subsequent studies, however, with special techniques, revealed that the capsular material does have some influence on the pathogenicity of these bacteria.

Antiphagocytic Effect of the M Substance and Hyaluronic Acid Capsule. In order to analyze the method of destruction of these streptococci in the

animal body, an *in vitro* test was employed, which involved phagocytosis of the streptococci by the leukocytes of normal human blood. Experiments with mouse and human blood revealed that the streptococci were phagocyted in the blood of both species. Although it was theoretically preferable to use mouse blood as a test system since mouse virulence was being studied, it was found necessary to employ human blood because of the difficulty of obtaining sufficient quantities of blood from mice. Furthermore, the use of human blood was in itself of interest because of the relationship of the virulence of group A streptococci to human infection. Therefore, normal children's blood, which contains no detectable natural opsonins for these microorganisms, was employed.

The crude bovine testicular extract used in all the experiments as a source of hyaluronidase was found, in agreement with earlier workers, not to impair the phagocytic function of the leukocytes. Moreover, this enzyme prevents the formation of capsules of group A and group C streptococci during their growth cycle (Figs. 11 and 12). The question arose as to whether this crude tissue extract might contain proteolytic enzyme capable of digesting the M antigen of the streptococcal cell. Experiments were devised to test this possibility.

Washed group A streptococcal cells, strain S23, matt, type 14, from 500 cc. of Todd-Hewitt broth culture, were suspended in 4 cc. of physiological saline and heat-killed at 56°C. for 30 minutes. To one-half of these cells was added 2 cc. of crude bovine testicular extract containing 200 viscosity-reducing units of hyaluronidase; to the other half was added the same amount of extract inactivated by heating for 30 minutes at 60°C. After incubation of these mixtures at 37°C. for 18 hours, M extracts were prepared, diluted in a twofold serial manner, and tested against homologous antiserum.

Each test sample revealed approximately the same amount of M substance on titration. The same experiment was repeated with the addition to the testicular extract of a reducing substance, thioglycollic acid, in a final concentration of 0.01 M, in order to activate any previously inactive proteolytic enzyme which might be present. Again the M titres obtained were essentially the same in the two systems. In an additional experiment, using M substance in solution, samples of M extract were mixed with an equal volume of testicular extract containing 20 viscosity-reducing units of hyaluronidase or with heat-inactivated testicular extract, and incubated at 37°C. for 18 hours. Twofold serial dilutions of the mixtures were made and tested against homologous antiserum. No evidence of digestion of M substance was obtained. Further studies also revealed that the crude bovine testicular extract failed to digest gelatin or casein. Since the testicular hyaluronidase employed did not digest the M protein on the streptococcal cell or in solution, this extract was considered suitable as a source of hyaluronidase free of proteolytic enzyme.

To determine the antiphagocytic effect of the M substance and hyaluronic acid capsule, opsonic index experiments were performed.

Suspensions of well encapsulated, 3 hour cultures of the glossy and matt variants were turbidimetrically standardized, and blood agar plates were poured to estimate the bacterial count. To each of a series of tubes were added 0.05 cc. of culture dilution containing approximately 1,000,000 streptococci, 0.25 cc. of fresh, heparinized, human blood, and 0.05 cc. of hyaluronidase (10 viscosity-reducing units), type-specific anti-M serum, or saline. After 20 minutes in a rotating machine at 37°C., the tubes were plunged into ice water to stop phagocytosis; and smears were made and stained with Wright-Giemsa solution. The per cent of active leukocytes and the number of cocci ingested by 100 polymorphonuclear neutrophils were determined.

TABLE III

Phagocytic Action of Normal Human Blood on Capsulated and Decapsulated Variants of Group A Streptococci

Variant	Glossy					Matt									
Strain	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type	1	3	12	14	19	1	3	12	14	19	1	3	12	14	19
Antibody	None					None					Anti-M Serum				
Capsulated streptococci															
No. of cocci phagocyted by 100 leukocytes	1596	1920	1664	1560	2060	39	26	45	46	40	1210	1560	1310	1240	1700
Percentage of active leukocytes	97	96	92	84	94	6	2	6	6	10	93	90	94	90	89
Streptococci decapsulated with hyaluronidase															
No. of cocci phagocyted by 100 leukocytes	2767	2355	2242	2210	2646	158	210	210	270	315	2340	2410	1780	1700	2710
Percentage of active leukocytes	99	93	93	92	96	12	24	20	12	25	95	97	95	91	93

The results of the foregoing experiment are illustrated in Table III. The antiphagocytic properties of the M substance are shown by the fact that the matt, M-producing variants of both capsulated and decapsulated streptococci are markedly more resistant to phagocytosis than the glossy non-M-producing variants. On addition of anti-M serum the encapsulated matt variants become as susceptible to phagocytosis as the encapsulated glossy variants. It will be noted that when hyaluronidase is added to the systems, with resulting removal of the capsules, more of the streptococci are phagocyted than in comparable systems in which the streptococci remain encapsulated because no hyaluronidase is added. These observations suggest that the hyaluronic acid capsule has some antiphagocytic property.

Photomicrographs of representative fields of smears made in the phagocytic experiments are shown for the strain S23, type 14 system. It should be noted that no phagocytosis occurs if M protein is synthesized by the streptococci regardless of whether the capsule is present (Figs. 13 and 14); however, it can be seen (Figs. 15 and 16) that phagocytosis of encapsulated, non-M-containing, glossy variants does take place, but that the decapsulated glossy variants are phagocyted to a somewhat greater extent. As can be noted in Fig. 15, after phagocytosis of encapsulated organisms the capsule is no longer visible, which suggested that the leukocytes may contain hyaluronidase capable of digesting the capsular material. However, efforts to isolate this enzyme from large amounts of leukocytes obtained from human blood were unsuccessful.

Because the ultimate fate of the streptococci ingested by the phagocytes is not shown by opsonic index experiments such as that just described, a bacteriostatic test was employed. By this method it has been demonstrated that the ingested bacterial cells are destroyed by the phagocytic leukocytes (16).

Tenfold serial dilutions were used of a 3 hour broth culture of well encapsulated streptococci, ranging from 10^{-1} through 10^{-6}. In these experiments, 200 to 300 bacterial cells were contained in the 10^{-6} dilutions. To each of a series of tubes were added 0.05 cc. of culture dilution, 0.05 cc. of hyaluronidase (10 viscosity-reducing units), saline, or type-specific anti-M serum, and 0.25 cc. of fresh, heparinized, human blood. After incubation at 37°C. in a rotator for 3 hours, samples from each mixture were removed and streaked on rabbit blood agar plates. The resulting growth after 18 to 24 hours' incubation at 37°C. was recorded on a ++++ to 0 scale.

Typical experiments are shown in Table IV, which reveals that the encapsulated, glossy variants are extremely susceptible to phagocytosis and fail to survive in the blood; on the other hand, the matt variants are resistant. In the presence of homologous anti-M serum these matt variants become as susceptible as the glossy variants. When hyaluronidase is added, which keeps the streptococci from regenerating their capsules throughout the 3 hour experimental period, it can be noted that the decapsulated matt variants retain their resistance to the bacteriostatic action of the blood. The decapsulated as well as the encapsulated matt variants become as susceptible on inclusion of anti-M serum in the system as the glossy variants.

It appears from the findings of these *in vitro* experiments that the M substance is far more important than the hyaluronic acid capsule in causing the streptococci to resist the phagocytic and bacteriostatic effect of the blood, but there was a definite indication from the opsonic index experiments that removal of the capsule enhanced the phagocytic effect of the leukocytes.

Demonstration of Protective Capacity of Crude Bovine Testicular Hyaluronidase against Group C Streptococcal Infection in Mice.—Although the rôle played by

TABLE IV

Bacteriostatic Action of Normal Human Blood on Capsulated and Decapsulated Variants of Group A Streptococci

Variant.....	Glossy					Matt									
Strain.....	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19	T1	T3	NY5	S23	T19
Serological type.	1	3	12	14	19	1	3	12	14	19	1	3	12	14	19
Antibody.....	None					None					Anti-M serum				
Capsulated streptococci															
Dilution of culture															
10^{-1}	++++	++++	+++	+++	+++	++++	++++	++++	++++	++++	++++	++++	+++	+++	++++
10^{-2}	+±	++±	+	+	++	++++	++++	++++	++++	++++	+±	+++	++	++	+++
10^{-3}	+	7	0	+	++	++++	++++	++++	++++	++++	+	4	6	1	+
10^{-4}	9	0	0	0	3	++++	++++	++++	++++	++++	2	2	4	0	6
10^{-5}	0	0	0	0	0	++++	++++	+++	++++	++++	0	0	0	0	1
10^{-6}	0	0	0	0	0	+++±	++++	+++	+++	++++	0	0	0	0	0
Streptococci decapsulated with hyaluronidase															
Dilution of culture															
10^{-1}	++++	++++	+++	+++	+++	++++	++++	++++	++++	++++	+++	+++	++++	++++	++++
10^{-2}	++	+	9	9	+±	++++	++++	++++	++++	++++	+	++	+	++	+++
10^{-3}	+	2	0	0	+	++++	++++	++++	++++	++++	+	0	7	1	+
10^{-4}	0	0	0	0	0	++++	++++	+++	++++	++++	0	0	0	0	7
10^{-5}	0	0	0	0	0	++++	++++	+++	++++	++++	0	0	0	0	0
10^{-6}	0	0	0	0	0	+++	+++	+++	+++	+++	0	0	0	0	0

In bacteriostasis of strains, degree of growth is indicated on a ++++ to + scale; fewer than 10 colonies are represented in arabic numerals; 0 indicates no growth.

the M protein of the streptococcal cell in resisting the phagocytic action of leukocytes contained in human blood appeared clearly defined in the *in vitro* experiments, the effect of the capsule was still not well understood. We, therefore, attempted to study the problem in an *in vivo* system employing the mouse as a test animal.

To make certain that the preparation containing hyaluronidase was active in the *in vivo* system selected, the bovine testicular extract was tested for its effect in protecting mice against infection with group C streptococci. Previous workers, with one exception, obtained good protection in similar experiments.

The procedure employed was essentially the same as that described by Kass and Seastone (8) for protection experiments with group A streptococci. Mice were inoculated intraperitoneally with tenfold serial dilutions of 4 hour broth cultures of encapsulated group C streptococci, strain D181. The number of streptococci inoculated were calculated by pouring rabbit blood agar plates and the colony counts were 26 in the 10^{-7} dilution and 240 in the 10^{-6} dilution. Treatment with 0.5 cc. crude bovine testicular extract containing 100 viscosity-reducing units was started 2 hours later. Injections of enzyme were given intraperitoneally every 2 hours for the first 12 hours, every 4 hours during the next 36 hours, and every 12 hours for the last 48 hours; a total of 19 injections.

In Table V are illustrated the results of the protective influence of crude bovine testicular hyaluronidase on mice infected with group C streptococci. The enzyme protected all mice completely against 1,000 M.L.D. and some of the mice against even 100,000 M.L.D. A control group of mice treated with enzyme inactivated by heating to 60°C. for 30 minutes died as rapidly as the untreated virulence controls. These findings are remarkably similar to those of Hirst (4), who employed leech extract as a source of enzyme.

Method of Demonstrating Mouse Protection against Group A Streptococci with Hyaluronidase.—Since crude testicular hyaluronidase was thus found to be effective in protecting mice against infections with encapsulated group C streptococci (Table V), experiments were undertaken in an attempt to also obtain protection in mice with encapsulated group A streptococci. In the studies of Kass and Seastone (8), who were successful in such experiments, it was noted that the final dilutions of the streptococci were made in testicular extract and allowed to remain for 5 minutes at room temperature before injection of the mixture; streptococci used for the controls, however, were diluted in plain broth. Blundell (6) had previously shown that simultaneous injections of a mixture of bovine testicular extract and streptococci, followed by treatment with the enzyme, provided the greatest delay in the time of death as compared with untreated control mice. These observations suggested an explanation for the fact that we had earlier failed to obtain any protection of mice against group A streptococci since 2 hours had elapsed before enzyme treatment was started.

The effect of giving the hyaluronidase simultaneously with the streptococci

by diluting the bacteria in the testicular extract was compared with the effect of delaying treatment by diluting the streptococci in plain broth and giving the first injection of enzyme 2 hours later. The results of such an experiment are shown in Table VI. Encapsulated group A type 14 streptococci, strain S23, were employed. The colony count in the 10^{-7} dilution was 19 colonies and in the 10^{-8} dilution, 2 colonies. The treatment schedule was the same as that used against group C streptococci, except where earlier treatment was

TABLE V

Demonstration of Protective Activity of Crude Testicular Hyaluronidase

Mouse Protection against Group C Streptococci

Treatment of mice	Culture of streptococci, strain D181					
	cc.					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Unheated testicular hyaluronidase	D1	D2	S	S	S	S
	D2	S	S	S	S	S
	S	S	S	S	S	S
	S	S	S	S	S	S
Heated testicular hyaluronidase*			D1	D1	D1	D1
			D1	D1	D1	D2
			D1	D1	D2	D2
			D1	D2	D2	D2
Untreated virulence controls	D1	D1	D1	D1	D1	D1
	D1	D1	D1	D1	D1	D2
	D1	D1	D1	D2	D2	D2
	D1	D1	D1	D2	D2	D2

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

* Hyaluronidase heated at 60°C. for 30 minutes.

given by diluting the cultures in hyaluronidase. In diluting the streptococci in hyaluronidase, the concentration of enzyme was adjusted so that the mouse received the same amount of enzyme as that used in the later therapeutic doses; *i.e.*, 100 viscosity-reducing units.

When the streptococci were diluted in plain broth and the mice subsequently treated after a lapse of 2 hours with 19 injections of hyaluronidase for 96 hours, only a slight delay in the rate of death was obtained as compared with the untreated controls. In contrast, when the streptococci were diluted in a solution of hyaluronidase, even though no additional treatment was given, there was some protection in the 10^{-8} dilution and a delay in the time of death in the other dilutions. Moreover, when the mice, in addition to the first dose

TABLE VI

Method of Demonstrating Protective Action of Crude Testicular Hyaluronidase against Group A Streptococci

Mouse Protection Test

Treatment of mice		Culture of streptococci, strain S23 matt, type 14				
		cc.				
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Streptococci diluted in plain broth	Untreated virulence controls	D1	D1	D1	D2	D3
		D1	D1	D1	D2	D3
		D1	D1	D1	D2	D3
		D1	D1	D2	D2	D3
		D1	D1	D2	D2	D3
		D1	D2	D2	D3	D4
	Treated with multiple injections of hyaluronidase	D1	D1	D2	D3	D3
		D1	D1	D2	D3	D3
		D1	D1	D2	D3	D4
		D1	D2	D3	D3	D4
		D2	D2	D3	D3	D4
		D2	D2	D4	D4	S
Streptococci diluted in solution of hyaluronidase	No additional treatment	D1	D2	D3	D4	D5
		D1	D2	D3	D4	D5
		D2	D2	D3	D4	D5
		D2	D2	D3	D5	S
		D2	D2	D4	D5	S
		D2	D2	D4	D5	S
	Treated with multiple injections of hyaluronidase	D1	D1	D4	D5	S
		D1	D2	D5	S	S
		D1	D3	D5	S	S
		D2	D3	D6	S	S
		D2	D3	D6	S	S
		D2	D3	S	S	S

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

Duplicate sets of mice treated with hyaluronidase heated at 60°C. for 30 minutes all died at the same rate as untreated virulence controls.

of hyaluronidase, were also treated with multiple injections (19 injections for 96 hours), protection was obtained against 10 M.L.D., with marked delay in the time of death of the other mice, as compared with the untreated virulence controls.

It is thus apparent that protection against group A streptococcal infection in mice can be obtained when hyaluronidase is given concomitantly with the

TABLE VII

Combined Action of Antiserum and Crude Testicular Hyaluronidase in Protecting Mice against Infection with Group A Streptococci

Mouse Protection Tests

Treatment of mice		Culture of streptococci, strain S23 matt, type 14							
		cc.							
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Antiserum alone	Single injection of antiserum given 16 hrs. before streptococci		D1	D3	S	S	S	S	S
			D1	D5	S	S	S	S	S
			D1	D6	S	S	S	S	S
			D2	S	S	S	S	S	S
			D3	S	S	S	S	S	S
			D3	S	S	S	S	S	S
Testicular hyaluronidase alone	Multiple injections of hyaluronidase*					D3	D3	D7	S
						D3	D3	S	S
						D3	D3	S	S
						D3	D3	S	S
						D3	D3	S	S
						D3	D4	S	S
Both antiserum and testicular hyaluronidase	Each given as above*	D2	S	S	S	S			
		D3	S	S	S	S			
		D3	S	S	S	S			
		D3	S	S	S	S			
		S	S	S	S	S			
		S	S	S	S	S			
Control: antiserum and saline	Antiserum as above: multiple injections of saline instead of hyaluronidase		D1	D2	S	S			
			D1	D5	S	S			
			D1	S	S	S			
			D2	S	S	S			
			D2	S	S	S			
			D2	S	S	S			
Untreated virulence controls			D1	D1	D1	D1	D2	D2	D2
			D1	D1	D1	D1	D2	D2	D4
			D1	D1	D1	D2	D2	D2	D4
			D1	D1	D1	D2	D2	D2	D4
			D1	D1	D2	D2	D2	D2	D5
			D1	D1	D2	D2	D2	D4	S

S indicates survival of one mouse for at least 2 weeks.

D with numeral indicates death of one mouse within that number of days.

* Streptococci used for inoculation of these mice were diluted in hyaluronidase.

streptococci and followed by additional treatment with the enzyme, but not if the streptococci are diluted in broth and injected into mice, followed 2 hours later by treatment with hyaluronidase.

Combined Effect of Anti-M Serum and Hyaluronidase in Mouse Protection against Group A Streptococci.—Having demonstrated a method by which some protection of mice against encapsulated group A streptococci can be obtained by using hyaluronidase, thus confirming the work of Kass and Seastone (8), the protective effect of type-specific anti-M serum was compared with that of hyaluronidase; and the combined effect of these 2 agents was also investigated.

The method of treatment, which was demonstrated as being most effective in the preceding experiment (Table VI), was used. Five groups of mice were selected and each treated in a different manner. The first group was injected intraperitoneally with 0.5 cc. type-specific antiserum 16 hours prior to intraperitoneal inoculation with the streptococci, and no further treatment was given. The second group was inoculated with streptococci diluted in hyaluronidase and these mice were further treated with multiple injections of this substance as in the preceding experiment (Table VI). The third group received the combined treatment of the first two groups; *i.e.*, the mice received antiserum, were inoculated with streptococci diluted in hyaluronidase, and were further treated with multiple injections of the enzyme. The fourth group, used as a control for the effect of repeated injections, was treated with antiserum as in the first group, and received multiple injections of saline instead of hyaluronidase after inoculation of streptococci. The fifth group served as untreated virulence controls.

Table VII summarizes the findings in this experiment. With a single injection of antiserum, protection against 10,000 to 100,000 M.L.D. of group A streptococci was obtained. Protection against 10 M.L.D. was noted with hyaluronidase. With the combination of antiserum and testicular hyaluronidase there was an additive effect affording protection against 1,000,000 M.L.D., as compared with the untreated virulence controls. The control group, given antiserum and multiple injections of saline instead of hyaluronidase, revealed that there was no non-specific protective effect from the multiple injections.

The results of this experiment show that hyaluronidase, effective against the capsular material, and type-specific anti-M serum, specifically directed against the M substance of the streptococcal cell, each has a protective effect against group A streptococcal infection in mice. These findings also illustrate that the anti-M serum protects against 1,000 to 10,000 times as many M.L.D. as hyaluronidase and that the combined action of these two substances provides greater protection for mice than either one alone.

DISCUSSION

The protective effect of hyaluronidase derived from various sources has usually been unquestioned with regard to experimental group C streptococcal infections in laboratory animals, but conflicting evidence has been obtained by different investigators as to its protective action in group A streptococcal infections in mice. This has led to different concepts as to the influence of the hyaluronic acid capsule on the virulence of group A streptococci. Those workers, notably Hirst (4), who failed to protect mice against group A streptococcal infections with hyaluronidase, concluded from their studies that,

unlike group C streptococci, other factors such as the M antigen were more significant than the capsule in determining the virulence and invasiveness of group A streptococci.

The present work provides an explanation for the discrepancy between this point of view and that of Kass and Seastone (8) who, on finding some protection against group A streptococcal infection in mice treated intensively with hyaluronidase, expressed the view that the capsules of both group A and group C streptococci are responsible for the greater part of their virulence. Furthermore, these workers believed that the M antigen might be of only minor importance, since group A streptococci may elaborate M substance without necessarily being virulent for mice.

The evidence obtained in this study brings out the fact, well recognized by all investigators, that no single cellular component or product of group A streptococci, or indeed probably of any microorganism, contributes exclusively to the property of virulence. It is also recognized that factors still not known are essential to the development of virulence for different animal species. As an illustration, M-containing, encapsulated variants freshly isolated from patients, although evidently virulent for man are usually avirulent for mice. Apparently other cellular functions in addition to the production of capsular polysaccharide or M protein are also involved in the virulence of these microorganisms for mice. However, unless the strain elaborates the M antigen, it is not virulent for either mouse or man. Of the known factors, both the hyaluronic acid capsule and the M antigen of the streptococcal cell have been shown to contribute to the exhibition of virulence, and correspondingly the agents which lead to their destruction contribute to the protection of mice against infections with these streptococci. Thus, it has been amply demonstrated that the matt variants which elaborate the M substance are virulent for mice and resist phagocytosis; the glossy variants, however, which produce little or no M substance, are avirulent for mice and are susceptible to phagocytosis, even though both variants are encapsulated. This offers substantial evidence that the M substance is an important factor in the pathogenicity of group A streptococci.

In phagocytic experiments, on comparing the encapsulated streptococci and streptococci decapsulated with hyaluronidase, it was noted that the decapsulated bacteria were taken up by the leukocytes more readily and in larger numbers than the encapsulated forms. Further evidence was provided that the capsule is actually concerned with virulence to a certain extent by demonstrating that mice were protected against 10 M.L.D. of group A streptococci by hyaluronidase treatment.

To obtain this protective effect, it was essential to have the capsules removed by diluting the streptococci in a solution of hyaluronidase before injection and further to prevent capsule regeneration by frequent and continued

treatment with enzyme. No protection was obtained if the streptococci were diluted in broth and the multiple injections of hyaluronidase started 2 hours later, because the injected streptococci had multiplied so many times during the 2 hour interval that the enzyme therapy was inadequate to permit the host to deal with the increased number of bacteria. The technique of simultaneous injection of enzyme and streptococci explained the success of Kass and Seastone (8) in obtaining protection in mice; and it is likely that other workers also would have shown protective effects if such early and intensive treatment had been carried out.

The protection of mice could be increased from the 10 M.L.D. obtained by enzyme therapy alone to 1,000,000 M.L.D. by the combined use of anti-M serum and enzyme. A single injection of 0.5 cc. of antiserum 16 hours before inoculation of mice was sufficient to afford almost this much protection; and previous work indicates that even this dose of serum was a great excess (17). The greater protection afforded by the anti-M serum emphasizes the importance of the M substance in virulence of group A streptococci and is in agreement with other studies in this report dealing with the opsonic index, bacteriostatic, and mouse virulence experiments.

As previously demonstrated by Ward and Lyons (18) and shown in this report, encapsulation does not necessarily exclude phagocytosis. In the present experiments, decapsulated streptococci were slightly more susceptible to phagocytosis than those which retained their capsules. The encapsulated variants were nevertheless still highly susceptible to phagocytosis unless in addition to capsular substance they elaborated M antigen, whereas M-producing variants were only phagocyted to a very slight extent even though they were decapsulated with hyaluronidase. This suggests that encapsulation of group A streptococci and resistance to phagocytosis do not bear a direct relation to each other.

Though it is hazardous to generalize from these experiments in mice that the M substance and the capsular material play similar rôles in infections in man, the natural host for group A streptococci, it is worthy of note that one test system employed was dependent upon the phagocytic leukocytes in normal human blood. Both the M substance and the capsules had antiphagocytic properties against these human leukocytes. That the M protein is intimately related to virulence of streptococci for man is further supported by the fact that in this laboratory no strain tested and shown to lack M antigen has ever been isolated from a patient in the acute phase of streptococcal infection. In all strains tested, whenever the M substance could not be identified with the available type-specific antisera, it was possible by immunizing rabbits to show that the strain produced some other M substance not previously identified in this laboratory. On the other hand, strains have been isolated from patients in the acute phase of their infection which lacked

the capacity to produce the capsular polysaccharide; this is most strikingly illustrated in the case of strains of serological types 4 and 22 which produce hyaluronidase (19) and therefore do not develop capsules.

On the basis of the evidence presented, it appears that the hyaluronic acid capsule is one of the factors influencing virulence of group A streptococci, but that the M antigen appears to be a far more important factor in determining this property.

SUMMARY

Five strains of encapsulated group A streptococci of different serological types, each with a glossy and a matt variant, were studied to compare the rôles of the M substance and the hyaluronic acid capsule in virulence of these microorganisms. The results indicated that both contribute to the virulence of group A streptococci but that the M antigen is the more fundamental factor.

Encapsulated variants, both glossy and matt, were slightly less susceptible to phagocytosis than those from which the capsule had been removed with hyaluronidase. Glossy variants, containing no M substance, were readily phagocyted; matt, M-containing variants were resistant to phagocytosis except in the presence of anti-M serum when they became fully susceptible.

Only the M-containing, matt strains were mouse-virulent. Mice were protected against infections with these strains:

(*a*) By removal of the capsule with hyaluronidase, which resulted in slight protection, but only against 10 M.L.D. Early and intensive treatment was required to produce this effect; *i.e.*, simultaneous injection of enzyme and streptococci followed by prolonged enzyme therapy.

(*b*) By a single injection of anti-M serum administered the day before inoculation of the streptococci, which resulted in protection against 100,000 M.L.D.

(*c*) By combined use of enzyme and anti-M serum, an additive effect of the two protective agents occurred, which resulted in protection against 1,000,000 M.L.D.

The author wishes to thank Miss Grace Vanderhoff for technical assistance.

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EXPLANATION OF PLATES

These photographs were made by Mr. Joseph B. Haulenbeek.

PLATE 13

All preparations made with India ink; 3½ hour broth cultures of group A streptococci employed. × 1500.

FIG. 1. Capsule of glossy variant of type 1 (strain T1).

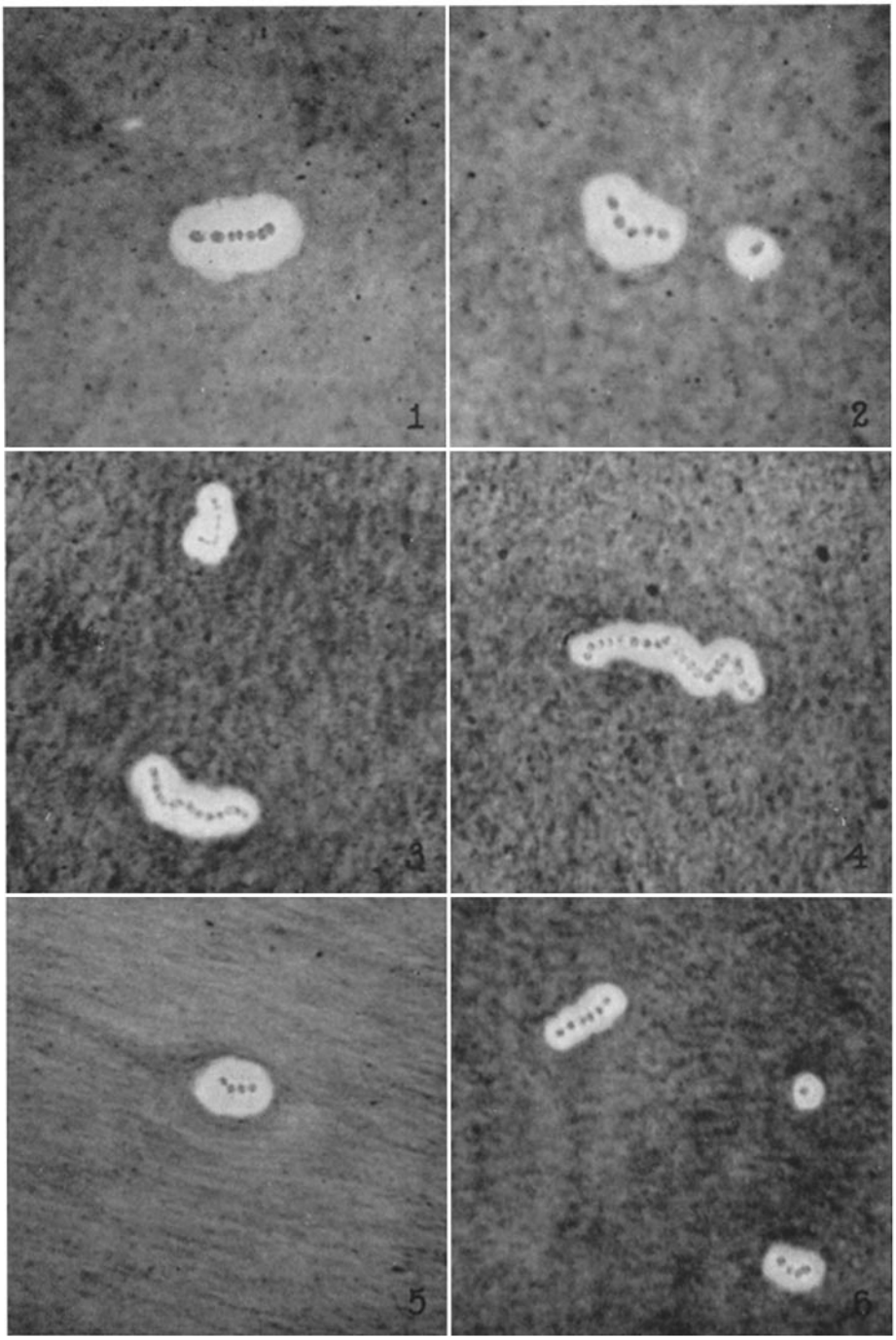
FIG. 2. Capsule of matt variant of type 1 (strain T1).

FIG. 3. Capsule of glossy variant of type 3 (strain T3).

FIG. 4. Capsule of matt variant of type 3 (strain T3).

FIG. 5. Capsule of glossy variant of type 12 (strain NY5).

FIG. 6. Capsule of matt variant of type 12 (strain NY5).



(Rothbard: Group A streptococcus infections)

PLATE 14

FIG. 7. Capsule of glossy variant of type 14 (strain S23).

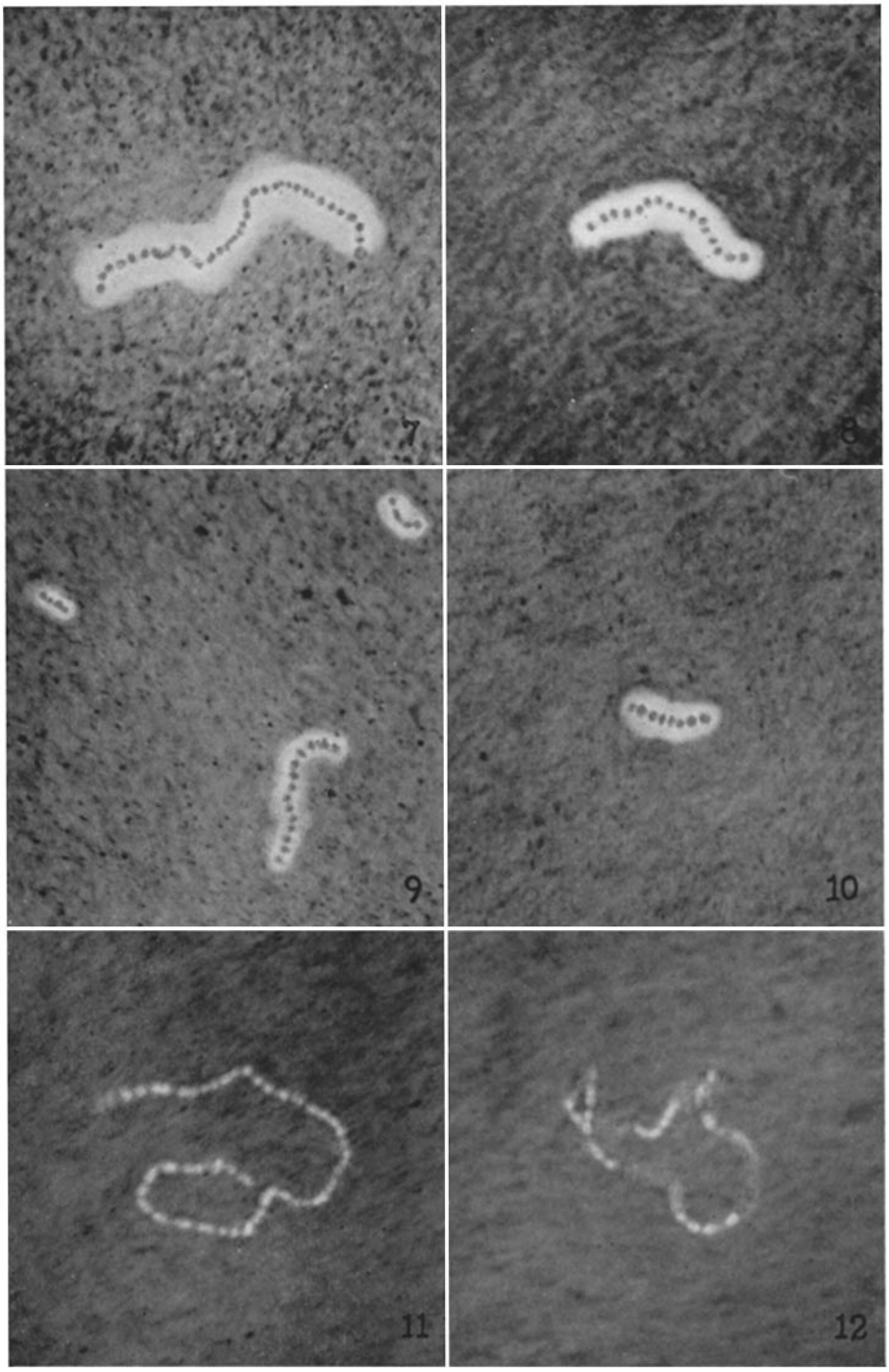
FIG. 8. Capsule of matt variant of type 14 (strain S23).

FIG. 9. Capsule of glossy variant of type 19 (strain T19).

FIG. 10. Capsule of matt variant of type 19 (strain T19).

FIG. 11. Decapsulated glossy variant of type 14 (strain S23); 3 minutes after addition of bovine testicular extract.

FIG. 12. Decapsulated matt variant of type 14 (strain S23); 3 minutes after addition of bovine testicular extract.



(Rothbard: Group A streptococcus infections)

PLATE 15

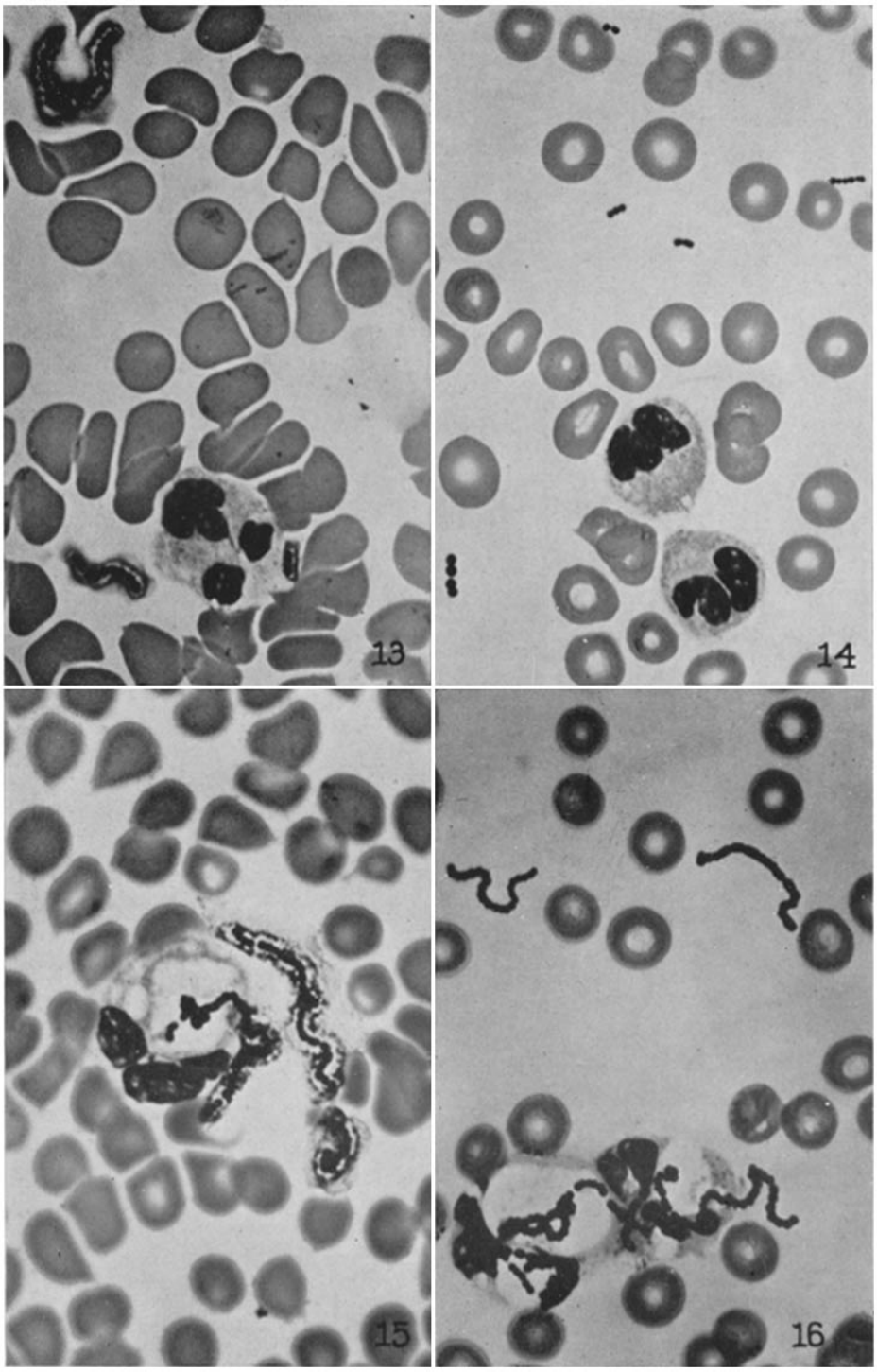
Films stained with Wright and Giemsa solution. These are representative specimens obtained from phagocytic experiment shown in Table III. × 1500.

FIG. 13. Capsulated matt variant of group A streptococci, type 14, (strain S23) not phagocyted by polymorphonuclear leukocyte.

FIG. 14. Decapsulated matt variant of group A streptococci, type 14, (strain S23) also not phagocyted by polymorphonuclear leukocytes. Bovine testicular extract employed in system to remove streptococcus capsule.

FIG. 15. Capsulated glossy variant of group A streptococci, type 14, (strain S23) being phagocyted by polymorphonuclear leukocyte. Note phagocytosis despite presence of capsule.

FIG. 16. Decapsulated glossy variant of group A streptococci, type 14, (strain S23) being phagocyted by polymorphonuclear leukocytes. Bovine testicular extract employed in system to remove streptococcus capsule. Note increased number of decapsulated streptococci being phagocyted as compared with encapsulated streptococci shown in Fig. 15.



(Rothbard: Group A streptococcus infections)