

STUDIES ON THE PATHOGENICITY OF GROUP A STREPTOCOCCI

I. ITS RELATION TO SURFACE PHAGOCYTOSIS*

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Streptococcus pyogenes is a highly ubiquitous organism which causes a variety of diseases in man (1). It has been found to elaborate during its growth "more extracellular toxic and enzymatic products than are recognized for any other human pathogen." (2) Despite the vast amount of knowledge which has accumulated concerning streptococci of one special kind (Group A hemolytic streptococci) the precise factors which determine their ability to initiate infection have remained obscure (1, 2).

In 1897 Bordet concluded that the principal defense of the host in streptococcal disease is phagocytosis (3, 4). His conclusions were reached as a result of meticulous observations made on peritoneal exudates from guinea pigs with experimental streptococcal peritonitis. In similar experiments performed in 1898 and 1899, Marchand (5) and Wallgren (6) noted that attenuated streptococci are readily destroyed by leucocytes, whereas virulent streptococci tend to resist phagocytosis.

Although the *in vivo* observations of Bordet, Marchand, and Wallgren, which related virulence to phagocytosis, have been confirmed (7), phagocytic experiments performed *in vitro* have yielded results which are difficult to interpret for the following reasons: (a) the strains of streptococci studied have often been inadequately characterized in regard to their content of cellular components known to be anti-phagocytic (7-9), (b) the tests have usually been performed in the presence of "normal" serum which may or may not contain "natural" opsonins (7-13), and (c) the cells used in the phagocytic tests have, with but rare exceptions, been obtained from species of hosts other than those in which the virulence has been measured (7-13). Recently Wiley and Wilson (12) have emphasized the failure of *in vitro* phagocytic tests, as customarily performed, to reveal phagocyte-bacterium relationships which correlate directly with streptococcal virulence.

Thus far only two factors have been clearly shown to affect the ability of Group A streptococci to *initiate* infection. The first of these, the antigenic M protein, has been

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extensively studied, particularly by Lancefield (14). Its presence on the surface of the streptococcal cell appears to render the organism resistant to phagocytosis (9, 11-14), and specific antibodies to it will protect the host (14). The second factor, the non-antigenic (1) hyaluronic acid capsule, has been less exhaustively investigated, but it too has been shown to possess antiphagocytic properties (9, 11, 13). When hyaluronidase is injected in sufficient quantities at the site of infection, a measurable degree of protection of the host may be demonstrated (9, 13). None of the other known components of the streptococcal cell, nor any of its numerous extracellular products have yet been demonstrated to influence the ability of the organism to cause infection (2).

Admittedly, the lack of correlation, which is so often observed between virulence and *in vitro* phagocytability, may be due to virulence factors which have not yet been defined and which do not affect phagocytosis. As has been suggested by Wiley and Wilson (12), however, the discrepancies may also be due to the fact that the methods, which have been used to study streptococcal phagocytosis *in vitro*, do not measure the ability of leucocytes to ingest streptococci *in vivo*. In the case of pneumococcal and Friedlander's bacillus infections just such a situation has been shown to exist (15-17). Pneumococcus Type I, for example, is highly resistant to phagocytosis in the absence of antibody when tested by the conventional glass slide or roller tube techniques, even though it is rapidly phagocytosed in the lung during the preantibody stage of infection. When, on the other hand, the laboratory phagocytic tests are modified to simulate more closely the actual conditions which obtain *in vivo*, phagocytosis of fully encapsulated pneumococci is readily demonstrated *in vitro*. The principal factors which determine the validity of phagocytic tests of this kind are: (a) the absolute concentration of leucocytes in the mixture, (b) the ratio of microorganisms to leucocytes, and (c) the availability to the leucocytes of suitable surfaces against which they can trap the encapsulated organisms (18). Since virulent pneumococci and Friedlander's bacilli are phagocytosed in the absence of antibody only when they can be trapped against such surfaces, the mechanism by which they are ingested has been termed "surface phagocytosis." It has been shown by direct observation to occur both *in vitro* and *in vivo* (19, 20).

The present studies were undertaken to determine the possible relationship of surface phagocytosis to the pathogenicity of Group A streptococci. As in the case of the pneumococcal and Friedlander's bacillus experiments (15-18), the *in vitro* phagocytic tests were performed in the absence of serum to avoid the possible effect of opsonins, and were carried out with washed leucocytes obtained from the same species of host in which virulence was measured. The *in vivo* observations were made in mice with acute streptococcal peritonitis, particular attention being paid to the earliest stages of the infection. The strains of streptococci selected for study were of known M content and were good producers of hyaluronate capsules.

Materials and Methods

Streptococcal Cultures.—Two pairs of Group A, Type 14 cultures were used in all experiments. The first pair consisted of the matt (M) and glossy (G) variants of Strain S23 (21).

This strain has been widely used in previous studies of streptococcal phagocytosis (9, 11, 13). The second pair included the matt virulent and matt attenuated variants of Strain T14 described by Hirst and Lancefield (22).¹ Stock cultures of all four strains were maintained in the lyophilized state on glass beads and were stored at 4°C.

The matt virulent variants, S23M and T14/46,² were subjected to frequent mouse passage to maintain virulence. All cultures in fluid media were made with freshly prepared beef infusion broth (23), enriched with 10 per cent sheep serum and 0.2 per cent dextrose, and adjusted to pH 7.8.³ Organisms cultured from the heart blood of mice were incubated in 4.5 ml. of broth overnight, were separated from the medium by centrifugation in the cold, and were resuspended in 1 ml. of fresh serum broth and stored in 0.1 ml. aliquots at -20°C. A separate aliquot was used to start the culture in each experiment. The S23 glossy variant and the avirulent matt variant, T14, on the other hand, were not subjected to mouse passage and were stored in defibrinated rabbit blood under vaseline at 4°C. (24). For the phagocytic and virulence tests, 0.05 ml. of either the frozen or the blood stock was inoculated in 4.5 ml. of serum dextrose broth and incubated overnight. An inoculum of 0.5 ml. of the primary culture was then added to a second tube of broth and incubated for 2 to 2½ hours (see below). Colony morphology of all strains was frequently checked on tryptose phosphate agar containing rabbit blood (5 per cent).

All four of the strains remained relatively stable throughout the period of study. Occasionally Strain S23M threw off a miniature hemolytic variant which was easily recognized on the blood agar plates and was readily eliminated by mouse passage. A non-encapsulated variant of S23G was also encountered on two occasions, and was found to be highly susceptible to phagocytosis even on glass (*vide infra*). In both instances it was eliminated by the preparation of new stock cultures from mucoid colonies. When a similar non-encapsulated variant was detected in an occasional culture of the matt attenuated strain, T14, new blood stocks, free of the variant, were prepared from the original desiccated culture on glass beads. Suspensions of the non-encapsulated M-containing variant could not be used in phagocytic tests because of their tendency to undergo spontaneous agglutination. (See also Fig. 1e of paper which follows.) No variants were noted in the cultures of T14/46.

Capsules.—Estimation of capsular size was made with India ink preparations as described by Butt *et al.* (25).

M Protein.—The M protein content of the cells of each strain was measured by the method of Lancefield (26). The extracts were made with 2 to 2½ hour cultures, and the precipitin tests were performed in capillary tubes (27) with Type 14 antiserum kindly supplied by Dr. Lancefield and by Dr. Elaine Updyke of the Communicable Disease Center in Atlanta.

¹ These cultures were kindly supplied by Dr. Rebecca Lancefield of The Rockefeller Institute. The matt-glossy terminology used in describing these strains is that of Lancefield (14) and refers to production of M protein rather than to colony formation. As indicated in Table I, all three of the matt strains produced M protein, whereas the glossy strain (S23G) did not. In keeping with the recent findings of Wilson (*J. Exp. Med.*, 1959, **109**, 257) the "glossy" strain, which produced large capsules, formed mucoid colonies when incubated in blood agar for 16 to 20 hours. After 24 to 48 hours the colonies assumed the matt form. As shown by Wilson these characteristics are associated with capsule formation and are unassociated with the production of M protein.

² This strain is designated "T14/46" throughout the study despite frequent mouse passages. The "46" refers to the number of times the parent T/14 strain had been passed through mice prior to the start of the present experiments.

³ Unless used within 3 to 4 weeks, when stored at 4°C., this medium does not support optimal growth of these strains of Group A streptococci.

Virulence Tests.—The LD₅₀ dose of each culture was determined for both mice and rats⁴ by intraperitoneal inoculations of serial dilutions of 2 to 2½ hour cultures. 6 mice and 6 rats were inoculated with each dilution of culture. The volume of inoculum used for mice was 0.2 ml. and that for rats 2.0 ml. All of the inoculated animals were observed for 7 days, and the LD₅₀ was calculated by the method of Reed and Muench (28).

Phagocytic Tests in Vitro.—Tests for phagocytosis *in vitro* were performed on glass slides, in roller tubes (29), and on the surfaces of fresh tissue (16) or moistened filter paper (16–18). The bacteria were harvested from 2 to 2½ hour cultures, and after having been washed twice in iced Locke's or gelatin-Locke's solution, the centrifugate was diluted in broth and counted in a Petroff-Hausser counting chamber. A volume of the centrifugate containing 4.5×10^8 streptococcal units⁵ was used for each phagocytic test.

Rat leucocytes were obtained from 21 hour peritoneal exudates produced by the injection of 5.0 ml. of a 1 to 1 mixture of starch-aleuronat and tryptose phosphate broth (16). The cells were washed from the peritoneal cavity of each of 4 or 5 rats with 15 ml. of iced gelatin-Locke's solution containing 1 mg. per cent of heparin. After being rewashed and pooled in a volume of 5 ml., the leucocytes were counted, and an aliquot containing 4.5×10^8 cells was centrifuged in the cold at 150 g for 5 minutes in a horizontal head. When the supernatant had been removed as completely as possible, an aliquot of washed bacterial centrifugate containing 4.5×10^8 streptococcal units was added to the packed leucocytes, and the volume of the mixture was adjusted to between 0.3 and 0.35 ml. with iced gelatin-Locke's solution. The final suspension, which contained a 1 to 1 ratio of streptococcal units and leucocytes (18), was thoroughly mixed and used immediately.

To obtain mouse leucocytes for similar suspensions, 0.1 to 0.15 ml. of the starch-aleuronat broth mixture was injected intraperitoneally in each of 25 mice. After 21 hours the peritoneal cavity of each mouse was washed with 5 ml. of heparin-gelatin-Locke's solution, and the total exudate was pooled and centrifuged at 150 g for 8 to 10 minutes. The remaining steps of the procedure were then carried out exactly as in the case of the rat leucocytes.

Tests for surface phagocytosis were performed as previously described (16–18). The streptococcus-leucocyte mixture (0.03 ml.) was spread evenly over a rectangular piece of filter paper (1 x 2 cm.) which had previously been placed on a glass slide and moistened with 0.015 ml. of gelatin-Locke's solution. The preparation was then put in a Petri dish, which was lined with moistened filter paper to prevent drying. After being sealed with adhesive tape, the Petri dish was placed in a 37°C. incubator for 30 minutes, at the end of which time the filter paper was removed to an iced Petri dish and washed repeatedly with 0.4 ml. of cold gelatin-Locke's solution. The leucocyte-streptococcus mixture thus obtained was centrifuged for 1 minute at 150 g and the supernatant, which contained most of the unphagocyted streptococci, was discarded. The centrifugate was resuspended in 0.15 ml. of normal salt solution containing 3 per cent gelatin, and four coverslip smears were made from the final suspension and were stained with methylene blue. The percentage of polymorphonuclear leucocytes containing one or more streptococcal units was determined by examining a total of 400 to 800 cells. Similar tests for surface phagocytosis were also performed with 1 x 2 cm. pieces of freshly excised liver, spleen and peritoneum (16).

Tests for phagocytosis on glass slides were done in the same manner. The leucocyte-streptococcus mixture (0.03 ml.) was spread evenly over a 1 x 2 cm. area of the slide before being incubated. Similar tests were also made with the standard roller tube method (29). The same volume of the leucocyte-streptococcus mixture was placed in a 7 x 44 mm. glass tube which

⁴ Webster Swiss mice weighing approximately 20 gm., and Wistar rats weighing 200 to 250 gm.

⁵ Each isolated coccus or diplococcus and each streptococcal chain was counted as a streptococcal unit (11–12).

was stoppered and rotated at approximately 9 r.p.m. during the 30 minute period of incubation.

Phagocytosis in Vivo.—25 male albino mice, weighing 20 gm. each, were injected intraperitoneally with 0.2 ml. of a 10^{-3} dilution of a $2\frac{1}{2}$ hour culture of S23M. Groups of 4 or 5 mice were sacrificed at intervals of 1, 2, 4, 12, and 18 hours after injection, and the peritoneal exudates from each group were separately pooled. The cells and fluid of the pooled exudates were then separated by centrifugation, and smears, stained with methylene blue, were made from both. The smears of the centrifugates were studied for phagocytosis, and those of the supernatant were examined to determine the approximate number of streptococci which had escaped ingestion by the phagocytic cells. Both the mononuclear and the polymorphonuclear leucocytes in the exudates were counted in the phagocytic tests (Table V).⁶

Three other groups of 25 mice each were injected respectively with: (a) the same dose of S23G, (b) 0.2 ml. of a 10^{-2} dilution of a 2 hour culture of T14, and (c) 0.2 ml. of a 10^{-2} dilution of a 2 hour culture of T14/46. The peritoneal exudates of each group were collected and handled exactly as described above for S23M.

EXPERIMENTAL RESULTS

Encapsulation and M Content of Cultures.—All four of the streptococcal strains used in these experiments (see Table I) formed easily demonstrable capsules when grown in beef infusion broth for 2 to $2\frac{1}{2}$ hours (Fig. 1). The capsules of S23M and S23G reached maximum size at the end of $2\frac{1}{2}$ hours, whereas those of Strains T14/46 and T14 were largest at 2 hours. Thereafter, the capsules of all strains became progressively smaller at approximately the same rate and were no longer demonstrable after 6 to 12 hours. When at their respective maximums, the capsules of the S23 strains were definitely larger than those of the T14 strains (see Fig. 1). These differences have been recorded in Table I on an arbitrary scale ranging from 0 to +++.

Large amounts of M protein were readily demonstrable in cultures of S23M, T14/46, and T14. No consistent quantitative differences were noted in the amount produced by S23M and T14/46, but both appeared to produce slightly more M antigen than did T14 (see Table I).⁷ Cultures of the glossy variant, S23G, on the other hand, contained no detectable M protein.

Comparative Virulence of Strains.—When injected intraperitoneally, Strains S23M and T14/46 were highly virulent for mice, whereas the glossy variant, S23G, and the matt avirulent Strain T14 were relatively avirulent. In rats the differences in virulence were similar though quantitatively less striking. The LD₅₀ data for all four strains are summarized in Table I.⁸

⁶ Although the mononuclear cells, particularly in the earliest exudates (1 hour), included some non-phagocytic lymphocytes, they were so outnumbered by the phagocytic monocytes and granulocytes as to have only a minor effect upon the percentage phagocytosis observed at each interval.

⁷ This same difference in the amount of M protein produced by T14 and T14/46 has been noted in precipitin dilution tests performed by Dr. Lancefield (30).

⁸ The statistical analyses included in Tables I, II, and IV were made with the assistance of Professor Margaret Merrell of the Department of Biostatistics.

Susceptibility to Surface Phagocytosis.—In order to determine the susceptibility of Group A streptococci to surface phagocytosis as compared to phagocytosis on glass, the S23 matt and glossy strains were tested for phagocytability on glass slides, in glass roller tubes, on the surfaces of freshly excised tissues and on moistened filter paper. The results obtained with rat leucocytes are summarized in Table II. Tests performed with mouse leucocytes yielded similar results. It will be noted that the matt strain, S23M, is resistant to surface phagocytosis as well as to phagocytosis on glass, whereas the glossy strain, S23G, is relatively susceptible to surface phagocytosis but resistant on glass (Fig. 2). Indeed, with

TABLE I
Comparative Properties of Group A Streptococcal Strains Used in Phagocytic Experiments

Strain	Type	Size of capsule	Production of M Protein	LD ₅₀ mice*	Log LD ₅₀ mice†	LD ₅₀ rats*	Log LD ₅₀ rats†
S23M (matt)	14	+++	+++	1	0	1.0×10^8	3.0181
S23G (glossy)	14	+++	0	1.4×10^5	5.1610	3.6×10^6	6.5481
T14/46 (matt virulent)	14	++	+++	1	0	2.1×10^6	5.3321
T14 (matt avirulent)	14	++	++	1.4×10^7	7.1400	5.8×10^8	8.7672

* Expressed in number of streptococcal units injected intraperitoneally (6 animals per dilution) and calculated according to the Reed-Muench method.

† Standard error for each log LD₅₀ mice is, in order, as follows: ± 0.44 , ± 0.52 , ± 0.34 , ± 0.34 ; and for each log LD₅₀ rats is: ± 0.55 , ± 0.35 , ± 0.51 , ± 0.28 .

phagocytic tests performed only on glass, it is not possible to differentiate between the highly virulent S23M strain and its avirulent variant, S23G. Tests for surface phagocytosis, on the other hand, readily distinguish the one from the other. Similarly, the matt virulent strain, T14/46, can be clearly differentiated from the matt avirulent strain, T14, by surface phagocytosis, whereas only slight differences at best are detectable between the two strains when the tests are performed on glass (see Fig. 3 and Tables III and IV).

Correlation of Virulence and Phagocytability.—The quantitative data relating to virulence and phagocytability *in vitro*, as tested on glass slides and on filter paper (surface phagocytosis), are shown in Tables III and IV. Table III depicts the results of experiments performed with mice; Table IV summarizes analogous experiments in rats. Both sets of data demonstrate a definite correlation between virulence and susceptibility to surface phagocytosis and an equally definite lack of correlation between virulence and phagocytability on glass.

Phagocytosis in Vivo.—The foregoing observations relating to virulence and

phagocytability *in vitro* suggest that surface phagocytosis may play a dominant role in antistreptococcal defense. To investigate this possibility further, a series of experiments was performed in which the evolution of streptococcal peritonitis was systematically studied in mice. Particular attention was paid to the earliest

TABLE II
Susceptibility of Strains S23M and S23G to Phagocytosis by Rat Leucocytes on Glass Slides, in Glass Roller Tubes, on Moistened Filter Paper, and on Surfaces of Freshly Excised Rat Tissues

Strain	Phagocytosis*					
	Glass slide	Glass roller tube	Filter paper	Liver	Spleen	Peritoneum
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
S23M (matt)	0	0	1	0	0	0
S23G (glossy)	0	0	28	17	26	37

* Percentage of polymorphonuclear leucocytes containing one or more streptococcal units.

TABLE III
Relation of Virulence of Streptococcal Strains in Mice to Their Susceptibility to Phagocytosis by Mouse Leucocytes on Moistened Filter Paper, on Glass Slides and in Glass Roller Tubes

Strain	LD ₅₀ -mice*	Phagocytosis†, §		
		Filter paper	Glass slide	Roller tube
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
S23M (matt)	1	2	0	1
S23G (glossy)	1.4×10^6	17	2	2
T14/46 (matt virulent)	1	3	1	1
T14 (matt avirulent)	1.4×10^7	67	9	17

* As in Table I.

† As in Table II.

§ Standard deviation (a measurement of the variability of the individual experiments) is for each experiment reading down the 3 columns in order, as follows: 0, 1.5, 0.45, 10.0; 0, 0.45, 0.32, 3.7; 0, 0.93, 0.32, 6.7.

stages of the infection. As is shown by the photomicrographs of Fig. 4 and the data in Table V, much less phagocytosis of the virulent strains (S23M and T14/46) occurs than of the avirulent (S23G and T14). The differences are particularly striking after only 1 hour, at which time monocytes dominate the exudate. Whereas the virulent organisms, which resist early phagocytosis,

grow out rapidly in the peritoneal fluid and eventually cause an overwhelming infection, the less virulent strains, which are promptly ingested, fail to get a

TABLE IV
Relation of Virulence of Streptococcal Strains in Rats to Their Susceptibility to Phagocytosis by Rat Leucocytes on Moistened Filter Paper, on Glass Slides, and in Glass Roller Tubes

Strain	LD ₅₀ rats*	Phagocytosis†, §		
		Filter paper	Glass slide	Roller tube
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
S23M (matt)	1.0 × 10 ³	3	1	1
S23G (glossy)	3.6 × 10 ⁶	28	1	0
T14/46 (matt virulent)	2.1 × 10 ⁵	10	1	2
T14 (matt avirulent)	5.8 × 10 ⁸	49	2	2

* As in Table I.

† As in Table II.

§ Standard deviation, recorded as in Table III, is for each experiment as follows: 1.8, 3.5, 0.53, 8.9; 0.58, 0.73, 0.62, 0.55; 0, 0, 0.60, 1.0.

TABLE V
Phagocytosis of Streptococci in Early Exudates of Mice with Streptococcal Peritonitis

Streptococcal strain	Phagocytosis*			Extracellular streptococci after 4 hrs.
	1 hr.†	2 hrs.†	4 hrs.†	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
S23M (matt)	0	1	3	Very many
S23G (glossy)	2	3	10	Few
T14/46 (matt virulent)	0	1	7	Many
T14 (matt avirulent)	9	13	14	Rare

* Recorded in terms of percentage phagocytosis as in Table II, except that counts included both mononuclear and polymorphonuclear leucocytes. Proportion of latter varied from 4 to 6 per cent at 1 hour, 12 to 36 per cent at 2 hours, and 40 to 55 per cent at 4 hours. Of the streptococcal units phagocytosed during the 1st hour more than 80 per cent were in monocytes; by the end of 4 hours the polymorphonuclear leucocytes not only had become predominant, but they also contained about 60 per cent of the ingested units.

† Time after inoculation.

satisfactory start in their growth and are soon destroyed. Since no inherent differences in the early growth rates of any of the strains could be demonstrated *in vitro*, it is concluded that the failure of the avirulent organisms to accumulate

as rapidly as the virulent ones is due to their more prompt destruction by the phagocytes.

From these observations it is evident that the outcome of the infection is determined within the first few hours and depends primarily upon the susceptibility of the infecting organisms to phagocytosis. That the monocytes normally present in the peritoneal cavity are the cells responsible for the primary phagocytic reaction is clearly shown by the data summarized in the footnote to Table V. The secondary wave of phagocytosis, on the other hand, is due to the polymorphonuclear leucocytes.

Since the *in vitro* observations previously described reveal that the avirulent strains, T14 and S23G, are both susceptible to surface phagocytosis on freshly excised peritoneum, whereas the virulent strains, T14/46 and S23M, resist surface phagocytosis under the same circumstance, it would appear reasonable to conclude that surface phagocytosis plays a critical role in determining the outcome of the peritoneal infection. The failure of the leucocytes to phagocytose the two virulent strains both *in vitro* and *in vivo* indicates that there is no appreciable amount of opsonizing type-specific antibody either on the surface of the excised peritoneum or in the peritoneal exudates of the infected animals.

DISCUSSION

The results of the present study are similar to those previously reported in relation to experimental infections caused by *Pneumococcus* type I and *Klebsiella pneumoniae* type A (15-20). Both of these organisms, even when fully encapsulated, have been shown to be susceptible to surface phagocytosis both *in vitro* and *in vivo*, in the absence of opsonins. Since Group A streptococci, like pneumococci and Friedlander's bacilli, behave essentially as extracellular parasites, in that they tend to be quickly destroyed when they have been phagocytosed (2, 13, 31), it is not surprising that the cellular defenses of the host should operate in much the same way in acute infections caused by all three of these organisms. The essential role played by surface phagocytosis in each instance appears to have been established.

Besides indicating the importance of surface phagocytosis as a mechanism of host defense in Group A streptococcal infections, the results of the foregoing experiments suggest that the principal factors, which determine the relative virulence of the four strains of streptococci studied, concern primarily the anti-phagocytic properties of the organisms. That these properties, when serum opsonins are rigidly excluded, cannot be properly assessed by the conventional phagocytic tests carried out on glass slides or in glass roller tubes is shown by the lack of correlation between the results of such tests and the virulence of each strain (see Tables III and IV). When the phagocytic tests are modified, however, to simulate more closely the conditions which obtain *in vivo* and which permit surface phagocytosis to occur, a relationship between virulence and

phagocytability is readily demonstrated. Nevertheless, since these phagocytic tests were performed in the absence of serum to assure the elimination of opsonizing antibody, they too may be said to have been "artificial." Studies designed to measure the possible effect of serum devoid of type-specific antibody are now in progress.

Wiley and Wilson (12) have shown that Group A streptococci, which are resistant to phagocytosis in the living state, retain their antiphagocytic properties when killed by exposure to heat or to mercury arc irradiation. This observation indicates that the extracellular products of living streptococcal cells are not of great importance in determining the phagocytability of Group A streptococci. Since the ability of each strain to initiate infection appears to be related primarily to its susceptibility to surface phagocytosis, it may be inferred that virulence, in this sense, is not directly determined by any known or hypothetical "toxin" of the streptococcal cell. Indeed, there is no conclusive experimental evidence that any of the numerous extracellular products, which have been identified, are responsible for the ability of virulent strains to *initiate* infection (2). This is not to say that one or more of them may not influence the course or even the nature of the resulting disease process once it has become established (1, 2).

The only factors of the streptococcal cell which are known to determine virulence, as just defined, are the M protein and the hyaluronate capsule (1, 2, 9, 11-13). The results of studies relating to the effect of each of these cellular components upon surface phagocytosis are reported in the paper which follows (32).

The critical effect of the earliest phases of tissue defense in limiting the ultimate spread of intradermal bacterial infections in guinea pigs has recently been demonstrated by Miles and his coworkers (33, 34). Employing a number of microorganisms of varying degrees of virulence, they have shown that the extent of the infection is determined at a relatively early stage following the "primary lodgement" of the bacteria. The present observations, concerning the evolution of experimental streptococcal peritonitis, suggest precisely the same conclusion. In the case of the streptococcal infections it has also been possible to demonstrate that the earliest defense reactions of the host involve surface phagocytosis, first by the monocytes (35) which are present in the normal peritoneal cavity, and later by the polymorphonuclear leucocytes which eventually dominate the inflammatory exudate.

Burke and Miles (34) have emphasized that much of the early destruction of bacteria in the skin precedes the arrival of substantial numbers of polymorphonuclear leucocytes at the site of the infection. The present studies reveal that the "preinflammatory" killing of streptococci in the peritoneal cavity is due to the mononuclear phagocytes that populate the normal peritoneal fluid. Similar observations relating to early phagocytosis by macrophages have also been

reported in experimental pneumococcal lymphadenitis (36) and in a variety of dermal lesions produced in mice (37).

Finally, it should be emphasized that the present studies deal only with the pathogenicity of four selected strains of Group A streptococci for mice and rats. Although it may be assumed that similar host-parasite relationships are involved in the initiation of human streptococcal infections, it is possible that other factors participate which have not been defined by these experiments. Because quantitative measurements of virulence cannot be readily made in human subjects, it will be difficult to obtain comparable data for human streptococcal infections.

SUMMARY

Four strains of Group A streptococci, possessing different degrees of virulence for both mice and rats, were tested for susceptibility to phagocytosis on glass slides, in glass roller tubes, and on the surfaces of freshly excised tissues and moistened filter paper. All of the tests were performed in the absence of serum to exclude the possible presence of opsonins. Only under conditions which allowed surface phagocytosis to take place was there a correlation between virulence and susceptibility to phagocytosis. A similar relationship between virulence and surface phagocytosis was also demonstrable *in vivo* during the early stages of experimental streptococcal peritonitis. Systematic study of the evolution of the peritonitis revealed that its outcome was determined by the phagocytic reaction which occurred in the first few hours of the infection.

BIBLIOGRAPHY

1. McCarty, M., The hemolytic streptococci, *in* Bacterial and Mycotic Infections of Man, (R. J. Dubos, editor), Philadelphia, J. B. Lippincott Co., 1958, chapter 10.
2. MacLeod, C. M., Pathogenic properties of bacteria and defense mechanisms of the host, *in* Bacterial and Mycotic Infections of Man, (R. J. Dubos, editor) Philadelphia, J. B. Lippincott Co., 1958, chapter 4.
3. Bordet, J., Contribution à l'étude du sérum anti-streptococcique, *Ann. Inst. Pasteur*, 1897, **11**, 177.
4. Bordet, J., A contribution to the study of antistreptococcus serum, *in* Studies in Immunity, (translated by F. P. Gay), New York, John Wiley and Sons, 1909, 108.
5. Marchand, L., Étude sur la phagocytose des streptocoques atténués et virulents, *Arch. Méd. exp. et anat. path.*, 1898, **10**, 253.
6. Wallgren, A., Experimentelle Untersuchungen über Peritoneale Infection mit Streptococcus, *Beitr. path. Anat. u. allg. Path.*, Jena, 1899, **25**, 206.
7. Ward, H. K., and Lyons, C., Studies on hemolytic streptococci of human origin; observations on virulent, attenuated and avirulent variants, *J. Exp. Med.*, 1935, **61**, 515.

8. Hare, R., Studies on immunity to hemolytic streptococci: III. Observations on the variations in resistance to phagocytosis displayed by broth cultures of strains of high and low virulence, *Brit. J. Exp. Path.*, 1931, **12**, 261.
9. Kass, E. H., and Seastone, C. V., The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of Group A hemolytic streptococci, *J. Exp. Med.*, 1944, **79**, 319.
10. Hare, R., On the phagocytosis of haemolytic streptococci of high and low virulence by the blood of patients infected with that organism, *Brit. J. Exp. Path.*, 1928, **9**, 337.
11. Morris, M., and Seastone, C. V., The relationship of M protein and resistance to phagocytosis in the beta hemolytic streptococci, *J. Bact.*, 1955, **69**, 195.
12. Wiley, G. G., and Wilson, A. T., The ability of Group A streptococci killed by heat or mercury arc irradiation to resist ingestion by phagocytes, *J. Exp. Med.*, 1956, **103**, 15.
13. Rothbard, S., Protective effect of hyaluronidase and type specific anti-M serum on experimental Group A infections in mice, *J. Exp. Med.*, 1948, **88**, 325.
14. Lancefield, R. C., Specific relationship of cell composition to biological activity, *Harvey Lectures*, 1940-41, **36**, 251.
15. Wood, W. B., Jr., Studies on the cellular immunology of acute bacterial infections, *Harvey Lectures*, 1951-52, **47**, 72.
16. Wood, W. B., Jr., Smith, M. R., and Watson, B., Studies on the mechanism of recovery in pneumococcal pneumonia. IV. The mechanism of phagocytosis in the absence of antibody, *J. Exp. Med.*, 1946, **84**, 387.
17. Smith, M. R., and Wood, W. B., Jr., Studies on the mechanism of recovery in pneumonia due to Friedlander's bacillus. III. The role of surface phagocytosis in the destruction of the microorganisms in the lung, *J. Exp. Med.*, 1947, **86**, 257.
18. Smith, M. R., and Wood, W. B., Jr., Surface phagocytosis. Further evidence of its destructive action upon fully encapsulated pneumococci in the absence of type-specific antibody, *J. Exp. Med.*, 1958, **107**, 1.
19. Smith, M. R., Perry, W. D., Berry, J. W., and Wood, W. B., Jr., Surface phagocytosis *in vivo*, *J. Immunol.*, 1951, **67**, 71.
20. Wood, W. B., Jr., Smith, M. R., Perry, W. D., and Berry, J. W., Studies on the cellular immunology of acute bacteriemia. I. Intravascular leucocytic reaction and surface phagocytosis, *J. Exp. Med.*, 1951, **94**, 521.
21. Dochez, A. R., Avery, O. T., and Lancefield, R. C., Studies on the biology of streptococcus. I. Antigenic relationships between strains of *Streptococcus hemolyticus*, *J. Exp. Med.*, 1919, **30**, 179.
22. Hirst, G. K., and Lancefield, R. C., Antigenic properties of the type-specific substance derived from Group A hemolytic streptococci, *J. Exp. Med.*, 1939, **69**, 425.
23. Zinsser, H., and Bayne-Jones, S., *A Textbook of Bacteriology*, New York, D. Appleton Century Co., 1939, 8th edition, 875.
24. Wood, W. B., Jr., Studies on the mechanism of recovery in pneumococcal pneumonia. I. The action of type-specific antibody upon the pulmonary lesion of experimental pneumonia, *J. Exp. Med.*, 1941, **73**, 201.

25. Butt, E. M., Bonyng, C. W., and Joyce, R. L., Demonstration of capsules about hemolytic streptococci with India ink or azo blue, *J. Infect. Dis.*, 1936, **58**, 5.
26. Lancefield, R. C., The antigenic complex of *Streptococcus hemolyticus*. I. Demonstration of a type-specific substance in extracts of *Streptococcus hemolyticus*, *J. Exp. Med.*, 1928, **47**, 91.
27. Swift, H. F., Wilson, A. T., and Lancefield, R. C., Typing Group A hemolytic streptococci by M precipitin reactions in capillary pipettes, *J. Exp. Med.*, 1943, **78**, 127.
28. Reed, L. J., and Muench, H., A simple method of estimating 50 per cent end-points. *Am. J. Hyg.*, 1938, **27**, 493.
29. Ward, H. K., and Enders, J. F., Analysis of opsonic and tropic action of normal and immune sera based on experiments with pneumococcus. *J. Exp. Med.*, 1933, **57**, 527.
30. Lancefield, R. C., personal communication.
31. Glaser, R. J. and Wood, W. B., Jr., Pathogenesis of streptococcal pneumonia in the rat, *Arch. Path.*, 1951, **52**, 244.
32. Foley, M. J., and Wood, W. B., Jr., Studies on the pathogenicity of Group A streptococci. II. The antiphagocytic effects of the M protein and the capsular gel, *J. Exp. Med.*, 1959, **110**, 617.
33. Miles, A. A., Miles, Ellen M., and Burke, J., The value and duration of defense reactions of the skin to the primary lodgement of bacteria, *Brit. J. Exp. Path.*, 1957, **38**, 79.
34. Burke, J., and Miles, A. A., The sequence of vascular events in early infective inflammation, *J. Path. and Bact.*, 1958, **76**, 1.
35. Sawyer, W. D., Smith, M. R., and Wood, W. B., Jr., The mechanisms by which macrophages phagocyte encapsulated bacteria in the absence of antibody, *J. Exp. Med.*, 1954, **100**, 417.
36. Smith, R. O. and Wood, W. B., Jr., Cellular mechanisms of antibacterial defense in lymph nodes. I. Pathogenesis of acute bacterial lymphadenitis, *J. Exp. Med.*, 1949, **90**, 555.
37. Tompkins, E. H., and Grillo, M. A., Factors favoring phagocytosis by reticulo-endothelial cells early in inflammation, *Am. J. Path.*, 1953, **19**, 216.

EXPLANATION OF PLATES

PLATE 36

FIG. 1. India ink preparations of 2 to 2½ hour cultures of streptococcal strains used in phagocytic studies. A—Strain S23M (matt). B—Strain S23G (glossy). C—Strain T14/46 (matt virulent). D—Strain T14 (matt avirulent). × 1,000.

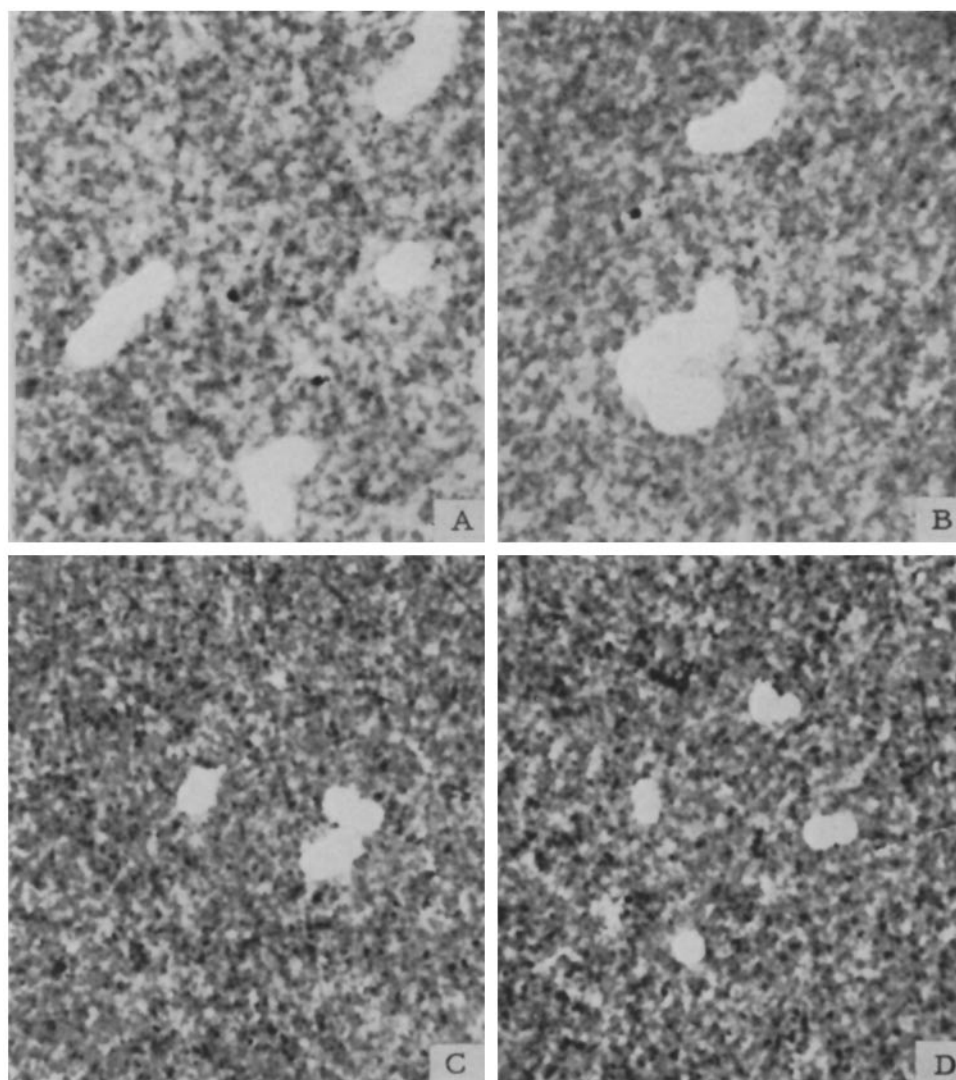


FIG. 1

(Foley *et al.*: Pathogenicity of Group A streptococci. I)

PLATE 37

FIG. 2. Phagocytosis of strains S23M (matt) and S23G (glossy) by rat leucocytes on glass slides (A—Strain S23M, B—Strain S23G), on moistened filter paper (C—Strain S23M, D—Strain S23G) and on freshly excised rat peritoneum (E—Strain S23M, F—Strain S23G). Most of the unphagocyted streptococcal units were removed by centrifugation before final smears were made. Note that S23M resists phagocytosis on all three surfaces, whereas S23G is phagocyted on filter paper and peritoneum, but not on glass. $\times 1,100$.

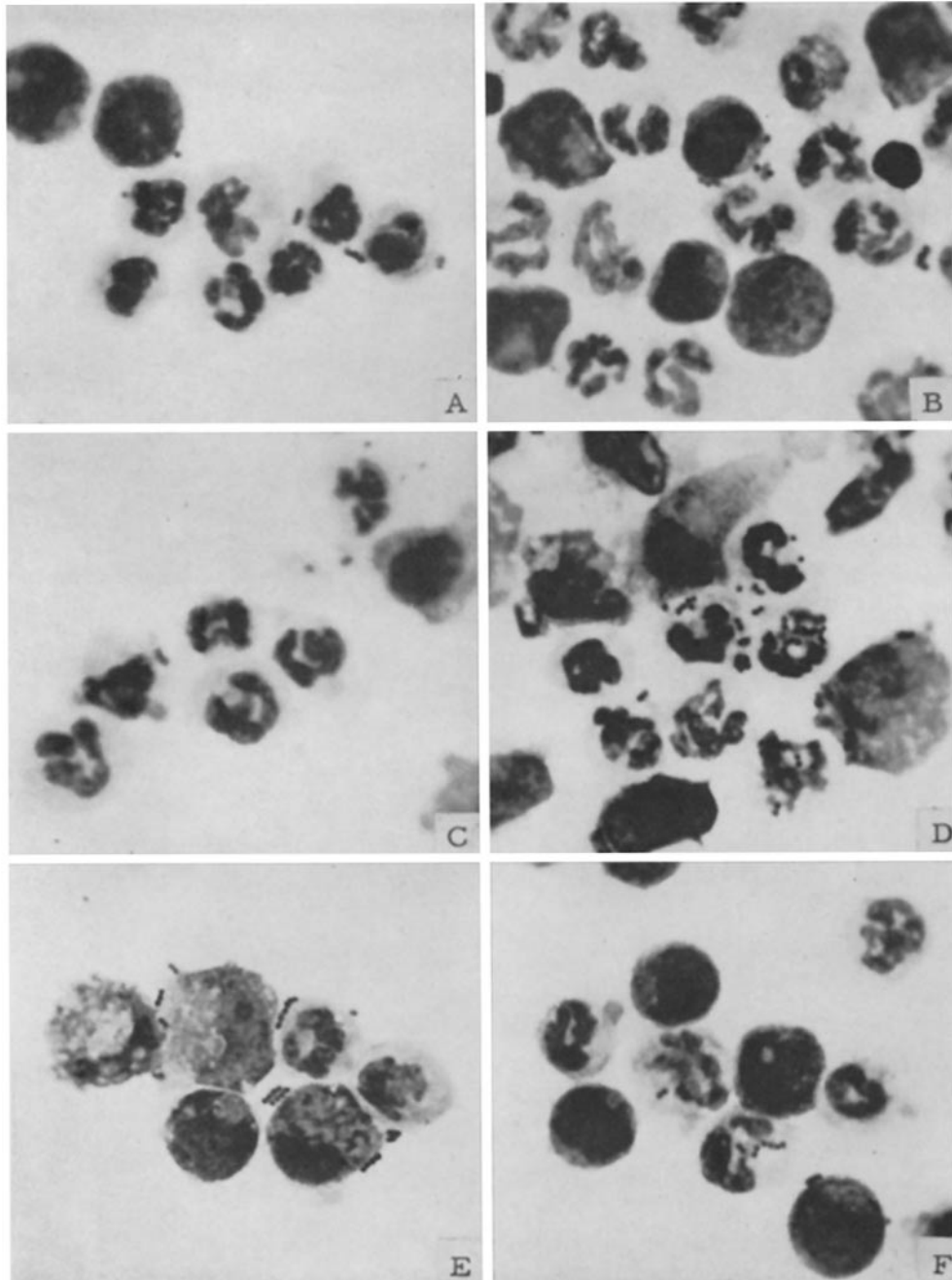


FIG. 2

(Foley *et al.*: Pathogenicity of Group A streptococci. I)

PLATE 38

FIG. 3. Phagocytosis of strains T14/46 (matt virulent) and T14 (matt avirulent) on glass slides and on moistened filter paper. Unphagocytosed streptococcal units were removed by centrifugation to facilitate counting of intracellular streptococci. Photomicrographs A (glass) and B (filter paper) show that T14/46 resists phagocytosis on both surfaces, whereas T14 is phagocytosed on filter paper (D) but not on glass (C). $\times 1,100$.

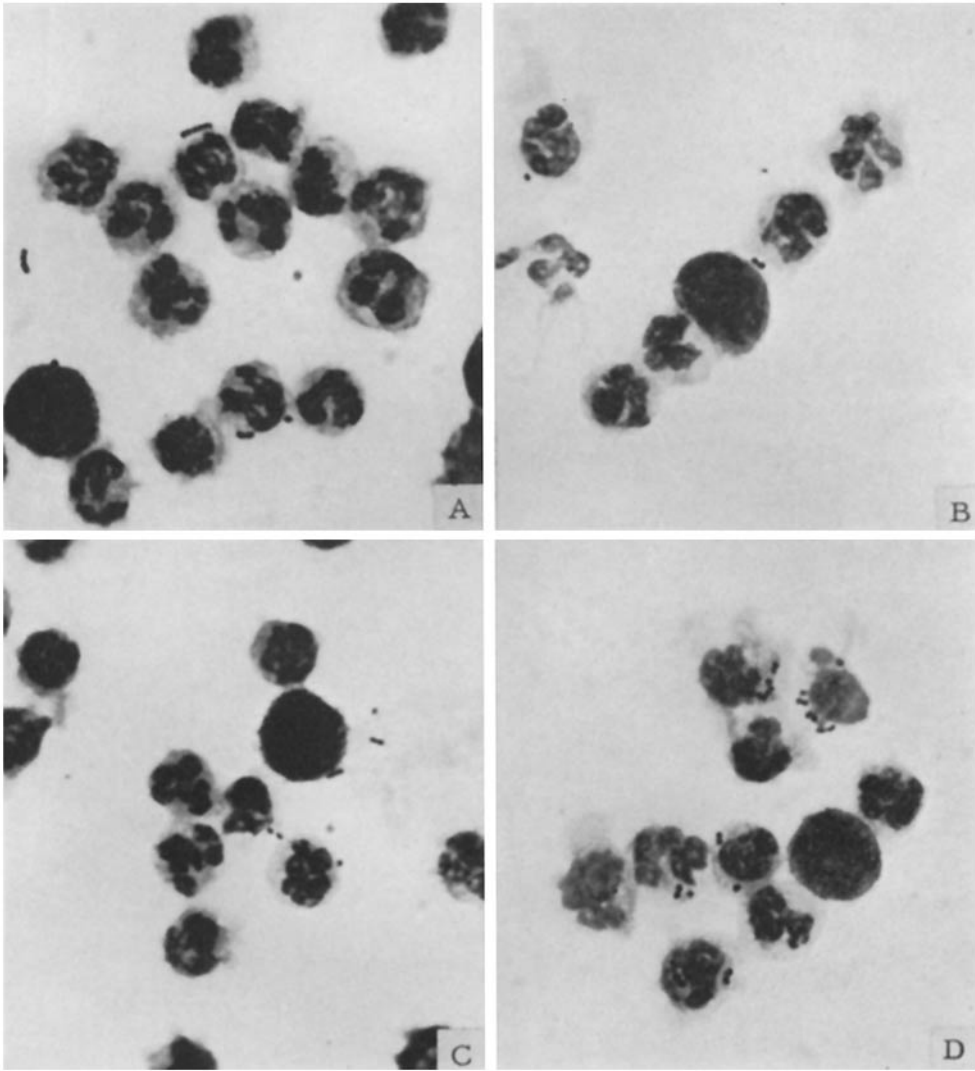


FIG. 3

(Foley *et al.*: Pathogenicity of Group A streptococci. I)

PLATE 39

FIG. 4. Smears of peritoneal exudates made at intervals of 1, 2, 4, 12, and 18 hours during course of peritoneal infection produced in mice by injections of T14 (matt avirulent) and T14/46 (matt virulent) streptococci. The T14 infection (left) rarely killed the mice, whereas the T14/46 infection (right) was uniformly fatal. Note phagocytosis of T14 organisms by monocytes in early stages of infection. $\times 1,100$.

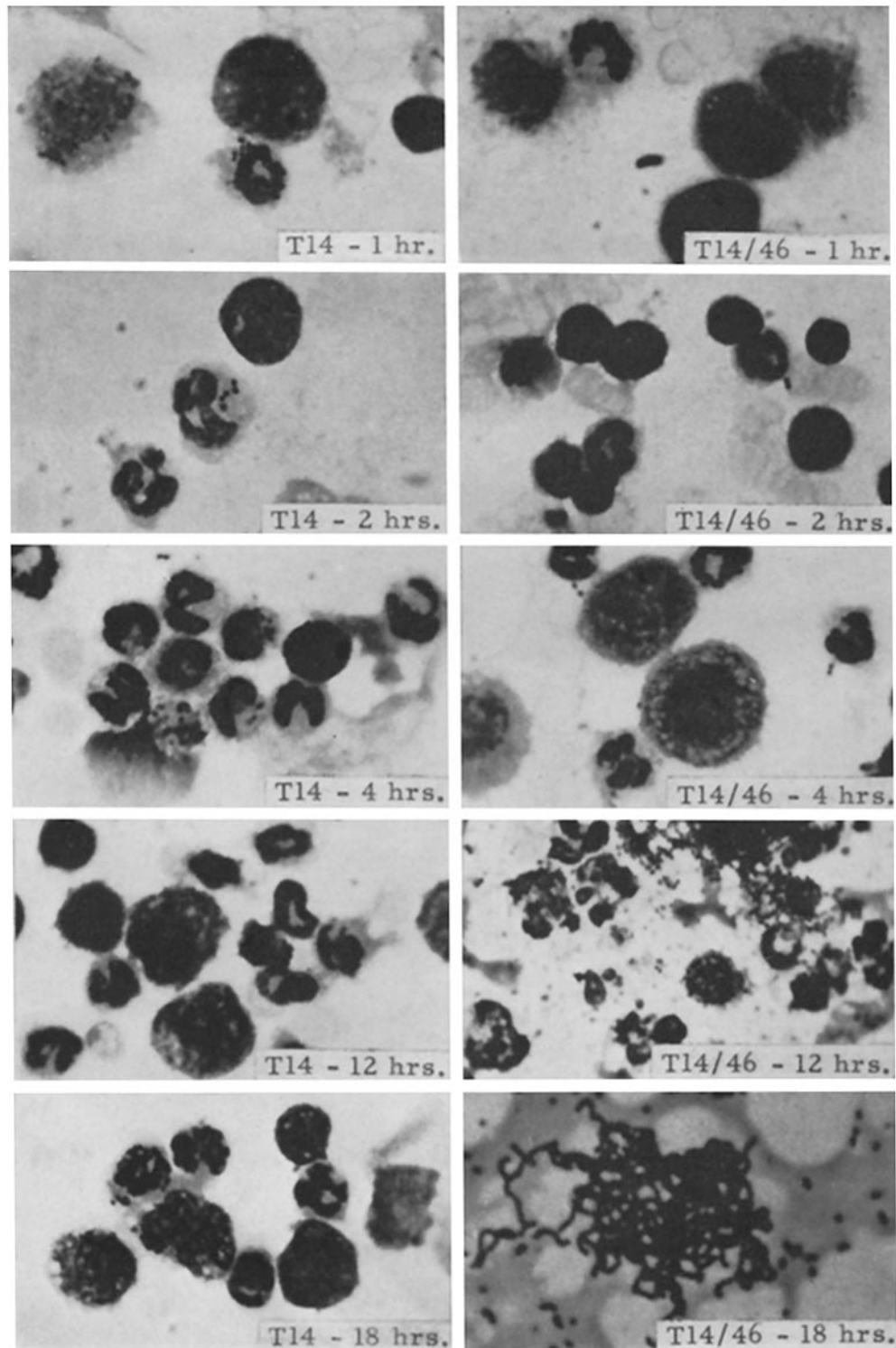


FIG. 4

(Foley *et al.*: Pathogenicity of Group A streptococci. I)