STUDIES ON THE PATHOGENICITY OF GROUP A STREPTOCOCCI

II. The Antiphagocytic Effects of the M Protein and the Capsular Gel*

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There is abundant experimental evidence that both the M protein and the capsular gel of Group A streptococci play significant roles in pathogenicity (1–7). That they enhance virulence by interfering with surface phagocytosis is indicated by the observations described in the preceding paper (8).

Despite the general agreement that both the capsules and the M protein of Group A streptococci are antiphagocytic (9, 10), their precise relationship to virulence has remained obscure. Numerous strains have been described, for example, which appear to produce abundant amounts of M protein and yet are relatively avirulent for mice (1, 11–14). Others possessing large capsules have, likewise, been found to be lacking in virulence (3, 15), as illustrated by the glossy variant of the S23 strain referred to in the accompanying paper (8). Indeed, it has been shown that streptococci which produce both capsules and M substance also may lack pathogenicity for mice. The T14 strain, used in this and the previous study, is just such an organism (8).

In the case of pneumococcal and Friedlander's bacillus infections, the situation is less complex (16), inasmuch as these organisms are protected against leucocytes by a single antiphagocytic factor (*i.e.*, a carbohydrate capsule) (9, 17, 18). As has been emphasized elsewhere, however, the quantitative aspects of capsule formation have a direct bearing upon the virulence of organisms such as Type III *Pneumococcus* (19, 20). This fact, taken in conjunction with the knowledge that a double antiphagocytic mechanism is involved in Group A streptococcal infections, has prompted us to restudy the *quantitative* and *combined* effects of the M protein and the hyaluronate capsule upon phagocytosis. The results appear to clarify the relationship of both factors to virulence.

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Materials and Methods

Streptococcal Cultures.—The four strains of Group A streptococci studied were the same as those used in the preceding experiments (8). They included: (*a*) the matt (M) and glossy (G) variants of Strain S23, both of which form large capsules, but only one of which (matt) produces detectable amounts of M protein (Table I), and (*b*) the matt virulent Strain T14/46 and its avirulent variant, T14 (Table II), both of which form capsules smaller than those of the S23 strains, and one of which (T14) produces less M antigen than the other (T14/46). The relative virulences of the four strains, when injected intraperitoneally in both mice and rats, vary over a relatively wide range and rank in the following order: S23M (most virulent), T14/46, S23G, and T14 (see Table I of previous paper (8)). The methods used in culturing these strains and in studying their capsules, their production of M protein and their susceptibility to phagocytosis have already been described (8). The phagocytic tests were performed with rat leucocytes.

Removal of M Protein.—Salt-free crystalline trypsin (lyophilized[1]) dissolved in Locke's solution at a concentration of 10 mg. per ml. was used in all experiments to remove M protein. As has been shown by Lancefield (21), this enzyme may be employed to destroy the M antigen without affecting the viability of streptococcal cells. Organisms from 2 to 2½ hour cultures, packed to a volume of approximately 0.4 ml. by centrifugation in the cold at 1500 *g* for 25 minutes, were suspended in 1 ml. of Locke's solution, to which was added 0.1 ml. of trypsin solution. As soon as the mixture had been thoroughly mixed and incubated for 12 minutes at 37°C., the organisms were washed with 5 ml. of Locke's solution to remove most of the trypsin and were centrifuged for 25 minutes at 1500 *g* in the cold. The packed streptococcal cells were then used immediately for phagocytic studies as previously described (8). Quadruplicate bacterial suspensions were prepared in each experiment. Suspension 1 served as a control, the organisms being incubated for 12 minutes in the absence of trypsin, and suspension 2 was incubated in the presence of trypsin. Both were used for phagocytic tests. Suspensions 3 and 4 were incubated in the presence and absence of trypsin, respectively, and were later tested with Type 14 antiserum (22) to determine whether or not all of the M antigen had been destroyed by the trypsin. No residual M protein was detected in any of the trypsin-treated suspensions.

Destruction of Capsular Gel.—Similar experiments were performed with streptococci pretreated with hyaluronidase to remove the capsular gel (5, 7). Lyophilized hyaluronidase[2] obtained from bovine testes was dissolved in Locke's solution in a concentration of 10 turbidity reducing units (TRU) per ml. A volume of 0.1 ml. (1 TRU) was added to 1.4 ml. of the bacterial suspension, prepared as described above, and the resulting mixture was incubated at 37°C. for 12 minutes. The enzyme-treated organisms were then lightly centrifuged at 400 *g* for 5 minutes, separated from the supernatant, and mixed in the usual way with leucocytes (8). To prevent the reformation of capsules (3, 5, 7) during the 30 minute period of incubation required for the phagocytic tests, 2 to 3 TRU of hyaluronidase in 0.02 to 0.03 ml. of Locke's solution were added to each mixture of leucocytes and enzyme-treated streptococci prior to the start of incubation.

Simultaneous elimination of M protein and capsular gel was accomplished by combining both of the above methods in a single procedure. When brought together, the two enzymes retained their activity during the period of incubation.

EXPERIMENTAL RESULTS

Effects of Enzymes upon Streptococcal Cells.—The incubation with trypsin invariably eliminated all detectable M antigen in the suspensions of the M-con-

[1] Worthington Biochemical Corporation, Freehold, New Jersey.

[2] Worthington Biochemical Corporation, Freehold, New Jersey.

taining Strains S23M, T14/46, and T14. Strain S23G, as already stated, produces no detectable M protein even in the absence of trypsin. Capsular studies performed with India ink (23) showed that the trypsin did not visibly alter the capsules of any of the four strains (see Tables I and II, and Fig. 1).

The treatment with hyaluronidase, on the other hand, had no significant effect upon the M protein but markedly reduced the size of all of the capsules (Tables I and II, and Fig. 1). When the concentration of enzyme was increased, or the period of incubation was prolonged, the organisms became completely denuded (Fig. 1) and spontaneous agglutination rapidly ensued. Since such unstable suspensions could not be used in the phagocytic tests (12), the hyaluronidase treatment was regularly stopped at the point at which a barely detectable amount of gel still remained on the surfaces of the organisms (Fig. 1). Even in this state, the treated streptococcal cells were often difficult to resuspend unless care was taken to avoid packing them during centrifugation (see Materials and Methods).

As expected, the combined treatment with trypsin and hyaluronidase removed both the capsule and the M protein (Tables I and II).

Effect of Enzymes upon Leucocytes.—To determine whether the trypsin, in the concentrations used, influenced phagocytosis by acting upon the leucocytes, phagocytic tests in both the presence and absence of trypsin were performed with the M-deficient Strain S23G. This strain was chosen for study because it lacks the protein antiphagocytic M substance upon which trypsin is known to act (21). Since the trypsin had no significant effect upon the results of the phagocytic tests (see Table I), it was evident that the enzyme neither stimulated nor depressed the phagocytic activity of the leucocytes. Similar experiments were done with hyaluronidase, using Type III pneumococci (19), the capsules of which are resistant to the action of the enzyme. The hyaluronidase was present in twice the concentration of that in the streptococcal phagocytic tests. That it likewise did not affect the leucocytes was indicated by the fact that the amount of phagocytosis which occurred was uninfluenced by its presence.[3] From these findings it may be concluded that neither of the enzymes, in the amounts used, acted directly upon the leucocytes. The effects that they did have in promoting phagocytosis in the experiments described below must, therefore, be attributed to their action upon the antiphagocytic components of the streptococcal cells.

Susceptibility of Enzyme-Treated Streptococci to Phagocytosis.—The results of the phagocytic tests with enzyme-treated organisms are summarized in Tables I and II.[4] The data reveal the following points of interest:

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[3] Hyaluronidase has been reported by Ludany *et al.* (24) to stimulate the phagocytosis of staphylococci and typhoid bacilli in the presence of serum, but the conditions of their experiments were quite different from those of the present study.

[4] The statistical analyses included in Tables I and II were made with the assistance of Professor Margaret Merrell of the Department of Biostatistics.

1. The phagocytic tests performed on filter paper (surface phagocytosis) are considerably more sensitive than those done with glass slides or roller tubes in detecting the changes in phagocytability resulting from the actions of the trypsin and the hyaluronidase. In the case of Strain S23M, for example, neither

TABLE I

The Effect of Trypsin and Hyaluronidase on Phagocytosis of Strains S23 Matt and S23 Glossy

Treatment	State of capsule*	State of M protein*	Phagocytosis‡, §		
			Filter paper	Glass slide	Roller tube
			per cent	*per cent*	*per cent*
Strain S23 matt					
None	+++	+++	3	1	1
Trypsin	+++	−	49	1	3
Hyaluronidase	±	+++	41	4	8
Trypsin plus hyaluronidase	±	−	64	29	40
Strain S23 glossy					
None	+++	−	28	1	0
Trypsin	+++	−	24	1	3
Hyaluronidase	±	−	74	25	58
Trypsin plus hyaluronidase	±	−	68	43	57

* Arbitrarily designated in terms of + and − symbols (8). In case of capsule, number of plus signs refers to approximate size of capsular envelope, and ± sign indicates removal of practically all of the capsule by hyaluronidase (see Fig. 1). The number of plus signs in the M protein column refers to the relative amount of the antigen demonstrable by precipitin test, and a negative sign denotes that none is detectable.

‡ Percentage of rat polymorphonuclear leucocytes containing one or more streptococcal units (8).

§ Standard deviation (a measurement of the variability of individual experiments (8)) is for each experiment, reading down the 3 columns in order, as follows: 1.8, 5.4, 2.8, 0.85, 3.5, 1.1, 3.6, 6.1; 0.6, 1.0, 1.9, 8.5, 0.73, 0.71, 16.0, 5.6; 0, 0, 4.1, 9.0, 0, 0.71, 7.0, 0.

the effect of the trypsin nor that of the hyaluronidase is clearly indicated by the results of the phagocytic tests on glass, whereas the effects of both are strikingly demonstrated by the tests on filter paper. The same is true also of Strain T14/46. Only when the antiphagocytic powers of the organisms are greatly reduced, does an appreciable amount of phagocytosis occur on glass, in

which case it is consistently somewhat greater in the roller tubes than in the "stationary" preparations on the glass slides.[5]

2. Removal of either the capsular gel or the M protein greatly increases the susceptibility of both the virulent strains, S23M and T14/46, to surface phago-

TABLE II

The Effect of Trypsin and Hyaluronidase on Phagocytosis of Strains T14/46 (Matt Virulent) and T14 (Matt Avirulent)

Treatment	State of capsule*	State of M protein*	Phagocytosis*, ‡		
			Filter paper	Glass slide	Roller tube
			per cent	*per cent*	*per cent*
Strain T14/46 (matt virulent)					
None	++	+++	10	1	2
Trypsin	++	−	52	0	1
Hyaluronidase	±	+++	33	5	8
Trypsin plus hyaluronidase	±	−	61	22	48
Strain T14 (matt avirulent)					
None	++	++	49	2	2
Trypsin	++	−	63	2	2
Hyaluronidase	±	++	52	36	50
Trypsin plus hyaluronidase	±	−	64	49	49

* As in Table I.

‡ Standard deviation, recorded as in Table I, is for each experiment as follows: 0.53, 1.3, 1.3, 6.3, 8.9, 0.71, 2.1, 0.71; 0,62, 0.57, 2.1, 8.9, 0.55, 0, 6.4, 3.5; 0.60, 0.99, 4.2, 8.1, 1.0, 0.71, 3.5, 3.6.

cytosis. Elimination of the two antiphagocytic factors simultaneously, by combined enzyme treatment, makes both strains still more susceptible to phagocytosis. Indeed, under the latter conditions, their susceptibility becomes so

[5] It is well known that the ingestion of easily phagocyted bacteria occurs more readily in a moving roller tube than on a stationary glass slide, owing to the greater number of contacts between leucocytes and bacteria resulting from the constant agitation of the roller tube mixture (25). It is often not appreciated, however, that no such difference is demonstrable in the case of encapsulated bacteria which are capable of resisting phagocytosis. This latter fact is well illustrated by the data in Tables I and II, relating to all of the untreated organisms.

great that they are readily phagocyted on glass. These observations clearly indicate that the antiphagocytic properties, and thus the virulence of Strains S23M and T14/46 depend upon the presence of both the capsular gel and the M protein.

3. As would be expected from the fact that the less virulent glossy strain, S23G, produces no detectable M substance, it (*a*) is not as resistant to surface phagocytosis (and thus not as virulent) as either S23M or T14/46, and (*b*) is rendered more susceptible to phagocytosis only by hyaluronidase. When its capsule is removed by the latter enzyme, the organism becomes so unresistant to phagocytosis that it is easily ingested even on glass.[6] What ability it possesses to protect itself against leucocytes appears, therefore, to be due primarily, if not solely, to its relatively large capsule.

4. A still different situation is exemplified by the least virulent strain, T14. Though it possesses both capsule and M protein, this strain has a definitely smaller capsule than either of the S23 strains and produces less M substance than its more virulent relative, T14/46 (8). That its smaller complement of M protein is functionally deficient is indicated by the facts: (*a*) that its destruction by trypsin causes little increase in phagocytability, and (*b*) that it possesses very little capacity to protect the organism against phagocytosis even on glass slides, once the capsule has been removed with hyaluronidase. The relatively small hyaluronic acid capsule, on the other hand, is somewhat more effective as an antiphagocytic factor, since it at least protects the organism from being phagocyted on glass.

DISCUSSION

These findings further substantiate the now generally accepted hypothesis (1–7, 9, 10, 12, 15) that both the M protein and the hyaluronic acid capsule are important virulence factors of Group A streptococci. In addition, they suggest a logical explanation for the quantitative differences in virulence exhibited by the four streptococcal strains which were subjected to analysis. Evidence that the virulence of each strain, as determined by intraperitoneal injection in mice and rats, is directly related to susceptibility to surface phagocytosis has been presented in the preceding paper (8). The results of the present study indicate that the phagocytability of each strain is dependent upon the combined antiphagocytic effects of the M protein and the capsular gel. The

[6] Similarly the pathogenicity of Group C streptococci, which produce large hyaluronate capsules but no detectable M substance, is markedly affected by treatment with hyaluronidase (3, 26).

The relative irregularity of the results obtained with the hyaluronidase-treated S23G strain on glass (note standard deviation, Table I) was due to the difficulty involved in removing exactly the right amount of the capsular gel with the enzyme (see Methods).

quantitative aspects of the combined effects have been shown to be of definite significance.

The data obtained from the phagocytic tests summarized in Tables I and II suggest the following conclusions:

1. The most virulent strain of the four studied, S23M (matt), owes its high degree of pathogenicity to the fact that it produces a relatively large capsule and a full complement of M protein. Both factors are strongly antiphagocytic.

2. The second most virulent strain, T14/46, also produces both factors, but its capsule is definitely smaller than that of S23M, and the combined antiphagocytic effects of its capsule and its M protein are not as pronounced as those of the more virulent S23M strain.

3. The third most virulent strain, S23G (glossy), produces no M substance, its pathogenicity depending primarily, if not entirely, upon the antiphagocytic effect of its large hyaluronic acid capsule.

4. The least virulent strain, T14, although its produces both a capsule and M protein, is deficient (as compared to S23M) in both factors, and particularly in the latter.

The reason for the relative lack of antiphagocytic effectiveness of the M protein in the T14 cell is not entirely clear. Besides being deficient in quantity, its location in the cell may be of importance (7, 15, 26). Since it is present in lesser amount than in T14/46, it may conceivably fail to extend far enough into the capsule to exert a maximum effect upon phagocytosis. Although the M protein is known to be concentrated primarily in the cell wall (27), it must be assumed to act either directly or indirectly upon the capsular surface, in order to influence the phagocytability of the encapsulated organism (7). It is evident, however, that more must be learned of the spatial relationship of the M protein to the hyaluronic acid capsule,[7] before the ineffectiveness of the T14 M substance as an antiphagocytic factor can be adequately explained. The one point that appears to be clearly established is that the lack of virulence of the T14 strain, for both mice and rats, is directly related to its inability to resist phagocytosis.

A number of attempts have been made to assess the relative importance of the hyaluronic capsule as a virulence factor in comparison to the M protein (3, 5–7). Although treatment of a number of strains of Group A streptococci with hyaluronidase has resulted in an increase of phagocytosis and destruction of the organisms *in vitro* (3, 5, 7), the results of analogous experiments performed *in vivo* have revealed only a slight effect from the hyaluronidase (3, 5, 29). The latter finding has led a number of workers to conclude that the hyal-

[7] The kind of information which may be needed is suggested by the studies of Tomcsik relating to the spatial relationships of the protein and carbohydrate components which form the capsules of certain Gram-positive bacilli (28).

uronic acid capsule contributes relatively little to the pathogenicity of the invading organisms (2, 3, 10). Such *in vivo* experiments must be interpreted with caution, however, since the actively metabolizing streptococcal cells will quickly form capsules as soon as the concentration of hyaluronidase in the surrounding medium falls below the level necessary for rapid depolymerization of the polysaccharide (3, 5, 7, 29). There is no assurance that the necessary level of enzymatic activity can be constantly maintained in the tissues by intermittent injections of the enzyme (5, 7). The protective effect achieved by the hyaluronidase treatment may, therefore, be a relatively poor indicator of the part played by the capsular gel. Indeed, the *in vitro* data reported in the present study clearly indicate that the hyaluronic capsules of Group A streptococci greatly enhance their capacity to resist surface phagocytosis. Since the defense of the host in the early stages of streptococcal infections has been shown to depend primarily upon surface phagocytosis (8), it must be concluded that the capsules play a significant role in pathogenicity. Manifestly both the M protein and the capsular gel are of great importance. When their *combined* actions are *quantitatively* measured by properly designed tests for phagocytosis, many of the discrepancies, which have recently been emphasized (10, 12, 26) regarding their individual roles in streptococcal virulence, appear to be explainable.

The double antiphagocytic effect of the M protein and the hyaluronic acid is strikingly similar to that of the "double capsule" of Type III *Pneumococcus* (19, 20). Virulent strains of the latter organism produce, in addition to the usual polysaccharide envelope, an outer "slime layer," which is present only during the logarithmic phase of growth, and which inhibits surface phagocytosis. When, during the later stages of growth, the slime layer diffuses from the Type III cell, leaving behind only the capsule proper, the organism becomes susceptible to surface phagocytosis, though it is still able to resist phagocytosis on glass. In this state it behaves like the S23M streptococcus which has been deprived either of its capsule or its M protein (see Table I). Thus both Type III pneumococci and Group A streptococci may exhibit three different degrees of susceptibility to phagocytosis. When Type III pneumococcus possesses its slime layer as well as its capsule proper, it is highly resistant both to surface phagocytosis and to phagocytosis on glass (19). The same is true of Group A streptococci, which produce large capsules and a full complement of M protein (8). When the streptococcus has lost either its M protein or its capsule, and the slime layer has diffused from the Type III capsule, both organisms are susceptible to surface phagocytosis but continue to be resistant on glass. Finally, if reduced to their "rough" forms, in which they possess neither of their antiphagocytic factors, both species are readily ingested by leucocytes even on glass. It is evident, therefore, that resistance to phagocytosis in the absence of opsonins is a relative matter and cannot be satisfactorily measured by phagocytic tests performed on glass slides or in glass roller tubes. Only when pneumococci and Group A streptococci are subjected to phagocytic tests which simulate

conditions *in vivo*, is there a meaningful correlation between phagocytability and virulence.[8]

One other similarity between Type III pneumococcus and Group A streptococci is noteworthy. It concerns their ability to produce suppuration. Unlike practically all other types of pneumococci, virulent strains of Type III not infrequently cause lung abscesses (30). Their occurrence can be readily demonstrated as a sequel to experimental Type III pneumonia in rats (20). Similar lesions occur following experimental streptococcal pneumonia (31), and suppuration is frequently observed in other experimental (32) and naturally occurring streptococcal infections (33). As reported elsewhere (20), the suppurative lesions produced by Type III pneumococcus are apparently caused by the extraordinarily high concentration of viable organisms which accumulate in the tissues. The ability of the Type III organism to reach such high population densities in the tissues is due to its ability to resist surface phagocytosis and thus to continue to multiply *in situ* for some time after the usual accumulation of tissue phagocytes. Other types of pneumococci, such as Type I, though relatively virulent, produce no demonstrable outer slime layer and accordingly are susceptible to surface phagocytosis (17). These organisms ordinarily reach a maximum population density in the tissues which is about a hundred times less than that reached by *Pneumococcus* III (20, 34). When, however, they are inoculated in sufficient numbers to produce a maximum tissue population comparable to that of Type III infections, they too cause suppuration (20). Since the most virulent strains of Group A streptococci are capable of resisting surface phagocytosis and thus of reaching relatively high population densities in the tissues (31), it would appear that the basic mechanisms involved in their suppurative capacities are similar to those which operate in lesions caused by *Pneumococcus* Type III.

SUMMARY

A quantitative study of the combined antiphagocytic effects of the M protein and the hyaluronic acid capsules of four strains of Group A streptococci revealed the following facts relating to their intraperitoneal virulence in mice and rats:

1. The most virulent strain, S23M (matt), produced both a large hyaluronic acid capsule and a full complement of M protein, the combined effects of which rendered the organism highly resistant to surface phagocytosis.

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[8] These statements should not be construed as indicating that all phagocytic tests performed on glass are valueless for estimating phagocytability, for manifestly they provide a rough measure of the ability of test organisms to resist ingestion. However, they do not give the leucocytes the same advantages that they have in tissues, and, therefore, the organisms appear to be more resistant to phagocytosis than they really are *in vivo*. Accordingly, it is not surprising that the results of tests on glass do not correlate as well with virulence as do the results of tests designed to measure surface phagocytosis.

2. The slightly less virulent strain, T14/46 (matt virulent) was somewhat more susceptible to surface phagocytosis owing to the fact that its smaller capsule was less antiphagocytic than that of the S23M organism.

3. The glossy variant of the S23 strain (S23G), which ranked third in virulence, was still more susceptible to surface phagocytosis because of its lack of detectable M substance. Its large hyaluronic acid capsule, however, was capable of protecting it against phagocytosis on glass.

4. The least virulent strain, T14 (matt avirulent), was the most susceptible of all to phagocytosis. Though it possessed both M substance and capsule, which together prevented its phagocytosis on glass, each of them was shown to be quantitatively and functionally deficient as compared to Strain S23M.

The differences in phagocytability, which appear to be directly related to the pathogenicity of the organisms, could be adequately demonstrated *in vitro* only by phagocytic tests designed to measure surface phagocytosis in the absence of opsonins. This fact is in keeping with the observation, previously reported, that surface phagocytosis plays a critical role in the defense of the host, particularly during the earliest stages of experimental streptococcal infections. Its possible relation to suppuration during the later stages of infection is also discussed.

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EXPLANATION OF PLATE 40

FIG. 1. India ink preparations of strain S23 matt showing effects of treatment with trypsin and with hyaluronidase. Organisms from 2½ hour cultures. × 1,100.

a. Untreated.

b. Treated with trypsin.

c. Treated sparingly with hyaluronidase. Note that capsules have not been completely removed.

d. Treated with hyaluronidase plus trypsin.

e. Overtreated with hyaluronidase. Such completely denuded organisms undergo spontaneous agglutination and are unsuitable for phagocytic tests.

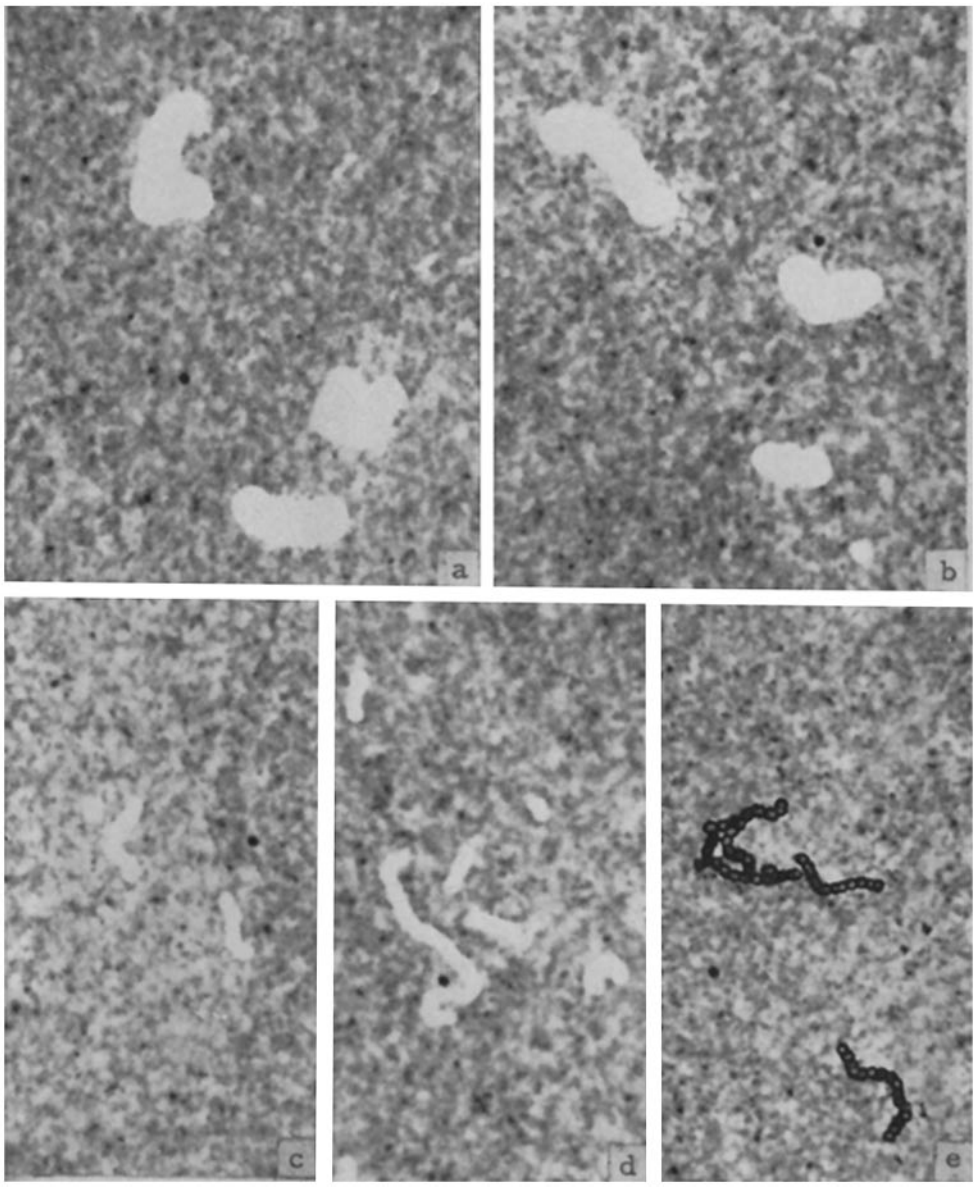

FIG. 1

(Foley and Wood: Pathogenicity of Group A streptococci. II)