STUDIES ON THE MECHANISM OF RECOVERY IN PNEUMOCOCCAL PNEUMONIA

III. Factors Influencing the Phagocytosis of Pneumococci in the Lung during Sulfonamide Therapy*

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Plates 15 and 16

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Studies of the action of sulfonamide chemotherapy upon the pulmonary lesion of experimental pneumococcal pneumonia in rats have shown that phagocytosis plays an important part in bringing about the final destruction of pneumococci in the lungs of treated animals (1). According to present concepts of immunology, the protective capsule of the pneumococcus renders it resistant to phagocytosis (2). It is generally believed that the fully encapsulated organism cannot be engulfed by phagocytic cells unless the surface of the capsule is altered by the presence of type-specific antibody (opsonin) or the capsule itself is sufficiently injured to destroy its antiphagocytic properties (3–5). If the phagocytosis of pneumococci is dependent upon one of these two conditions, there would appear to be only three likely explanations for the phagocytic reaction demonstrated in the pneumonic lungs of animals treated with sulfonamides. (1) The sulfonamide drug may injure the capsule of the pneumococcus either directly or indirectly and thus promote its phagocytosis. (2) The phagocytic reaction may be brought about by the presence of natural or acquired opsonins in the animal's serum. (3) A quantity of type-specific antibody sufficient to opsonize the pneumococci in the lung may accumulate locally in the pulmonary lesion although no opsonin can be demonstrated at the time in the circulating blood.

The experiments reported in the present paper have been designed to investigate each of these three possibilities.

Relation of Injury of the Pneumococcus Capsule to Phagocytosis

Pneumococci (A-5 strain) were grown in both rabbit blood broth and serum broth (1 per cent) (6) containing 10 mg. per cent of sulfapyridine. This concentration of drug caused

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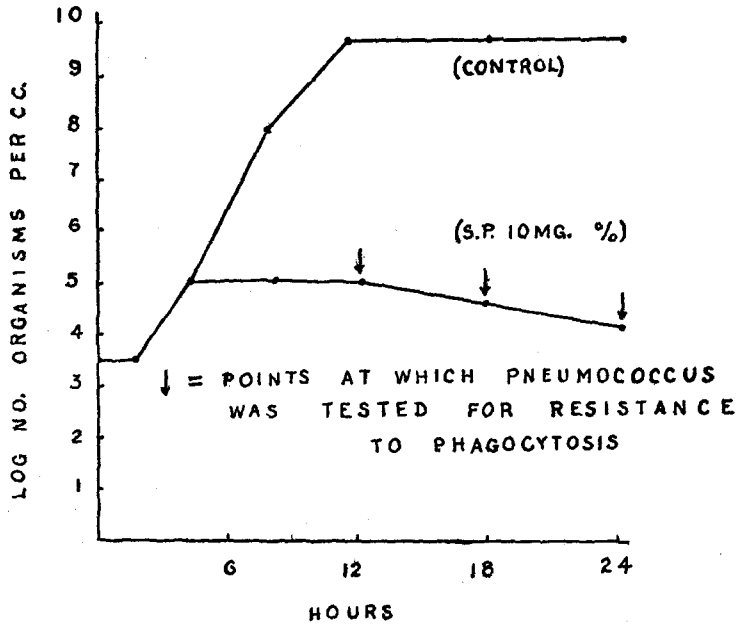
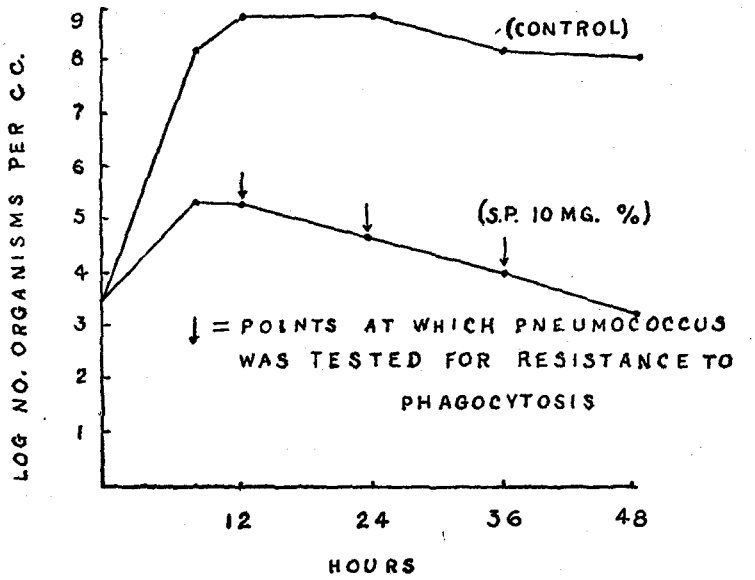


TEXT-FIG. 1. Effect of sulfapyridine on growth of pneumococcus in blood broth. S.P. = sulfapyridine.



TEXT-FIG. 2. Effect of sulfapyridine on growth of pneumococcus in serum broth. S.P. = sulfapyridine.

marked bacteriostasis, as shown by the growth curves in Text-figs. 1 and 2. Organisms were removed from the sulfapyridine broth cultures after 12, 18, 24, and 36 hours and were washed repeatedly in fresh broth; they were then added in various dilutions to tubes of normal, defibrinated rat blood, and were placed in a slowly rotating incubator according to the method of Chandler and Janeway (7). Tests for phagocytosis included (1) direct examination of blood films stained with methylene blue after 30 minutes, 1 hour, and 2 hours of incubation, and (2) determination of the viability of the organisms by culture at the end of 24 and 48 hours of incubation.

In all experiments the pneumococci which had been exposed to the action of sulfapyridine failed to be either phagocyted or killed by the leucocytes in the normal rat blood.[1] Since long exposure to the drug did not render the pneumococcus susceptible to phagocytosis, it can be concluded that the sulfapyridine itself does not injure the capsule of the organism sufficiently to promote phagocytosis.

Relation of Phagocytosis to Possible Circulating Opsonins

Type-specific opsonins might conceivably account for the phagocytic reaction observed in the lungs of animals receiving sulfonamide if immune bodies could be detected in rat serum either (1) as natural antibody present in the blood of normal rats, or (2) as acquired antibody accumulating in the serum during the course of the experimental infection. Repeated tests for natural antibodies against the A-5 strain of Type I pneumococcus were made with four of the most sensitive tests available for the detection of circulating immune bodies. The methods used included (*a*) the standard mouse protection test (8), (*b*) the standard bactericidal test (7), (*c*) an *in vitro* phagocytic test (5), and (*d*) a "modified" mouse protection test.[2] In none of the numerous experiments performed with these four methods was antibody detected in the serum of normal rats.

Since the phagocytic reaction seen in the lungs of rats recovering from experimental pneumonia occurs during the 2nd day of infection, it can be accounted for by acquired circulating type-specific opsonins only if these antibodies can be demonstrated as early as the 2nd day of the disease. As shown in Table I, type-specific antibodies do not appear in detectable quantity in the serum of rats recovering from experimental pneumonia until more than 3 days have elapsed.

[1] As is shown below, normal rat blood contains no demonstrable type-specific opsonins or bactericidal antibody for the strain of pneumococcus used in these tests. When antibody was added to the rat blood, however, pneumococci were promptly phagocyted and destroyed, indicating that the phagocytes in the blood were functionally intact.

[2] In the "modified" mouse protection test, pneumococci in the stationary phase of growth (5) were incubated for 4 hours in the test serum to insure opsonization. The serum-pneumococcus mixture was then inoculated intraperitoneally into mice having a preexisting sterile peritonitis produced by injection of starch-aleuronat. Each mouse received from 1 to 10 pneumococci.

It is concluded, therefore, that the phagocytosis of pneumococci in the lungs of rats recovering from experimental pneumonia is not related either to natural or to acquired type-specific opsonins occurring *in the blood serum.*

Relation of Phagocytosis to Possible Local Accumulation of Opsonins in the Lung

Pneumonic lungs of rats recovering from pneumococcal pneumonia (4th and 6th day) were frozen and ground in a ball mill or were minced in a Waring blendor and were then extracted with 15 per cent sodium chloride in order to split polysaccharide from any antibody which might be present (9). The polysaccharide was removed by dialysis and the protein precipitate was concentrated by dialysis under negative pressure. All mouse protection tests for antibody in such lung extracts were uniformly negative.

TABLE I

Mouse Protection Tests

Day of pneumonia on which blood was drawn from rats	Survival time* of *normal mice*‡ inoculated with rat serum (0.2 cc.) and pneumococci (10 organisms)					Survival time* of *prepared mice*‡ inoculated with rat serum (0.2 cc.) and pneumococci (10 organisms)				
Day 1	40	40	40	40	40	40	40	40	40	40
Day 2	40	40	40	40	40	40	40	40	40	66
Day 3	40	40	40	40	40	40	40	40	40	66
Day 4	S	S	S	S	S	S	S	S	S	S
Day 7	S	S	S	S	S	S	S	S	S	S
Controls (no serum)	40	40	40	40	66	22	40	40	40	40

* Number indicates time that mouse was found dead (hours after inoculation). S indicates survival for 1 week.

‡ Tests were done with both normal mice and mice "prepared" by injection of 0.8 cc. of 0.5 per cent sodium nucleinate 48 hours before the test to cause a sterile peritonitis.

Since efforts to extract antibody from the resolving pneumonic lung were unsuccessful, an attempt was made to demonstrate antibody *in situ* by testing for its presence in the pneumonic lesion. It seemed likely that antibody might be present in the lesion in sufficient concentration to opsonize pneumococci and yet in too small amounts to be detected by the relatively crude methods of chemical extraction (10).

Rats with Type I pneumonia were treated with sulfapyridine in the usual manner for 72 hours, at the end of which time all visible pneumococci in the lesion had been phagocyted and destroyed (1). Onto this resolving lesion was inoculated intrabronchially by cannula a second heavy suspension of virulent Type I pneumococci (0.5 to 1.0 ml. of an undiluted 6 hour broth culture) and the animals were sacrificed for histological examination of the lungs at intervals varying from 30 minutes to 4 hours after the second inoculation. As shown in the accompanying photomicrographs, phagocytosis of the second inoculum

of pneumococci begins within 1 hour (Fig. 1) and at the end of 4 hours practically all the organisms are found to be within the phagocytic cells (Fig. 2). The same results were obtained with animals treated for 96 hours before being given a second intrabronchial inoculation of pneumococci.

It was at first assumed that the phagocytic reaction occurring in the resolving lesion in the above experiments was due to type-specific opsonins and therefore would not occur when a heterologous type of pneumococcus was used in the second inoculation. This assumption was found to be incorrect, for when Type II pneumococci were inoculated upon a resolving (96 hour) lesion caused by a Type I organism (or *vice versa*) the same striking phagocytic reaction was observed. Fig. 3 shows the appearance of the phagocytic cells 4 hours after the second inoculation with a heterologous type of pneumococcus. It might be assumed that phagocytosis of the heterologous type of pneumococcus was due to the accumulation of circulating antibodies of the heterologous type during recovery. Mouse protection tests, however, showed that rats recovering from Type I pneumonia acquired no demonstrable immune bodies to the Type II pneumococcus. It was also suggested that the inoculation of pneumococci upon a resolving lesion might cause a very rapid production of antibodies corresponding in type to that of the second inoculum. But mouse protection tests done on the serum of rats bled at various intervals after the second inoculation revealed no such accelerated production of immune bodies.

To illustrate further the lack of type specificity of the phagocytic reaction observed in the resolving pneumonic lesion, an experiment was performed in which a Type I pneumonia was produced in the left lung and a Type II pneumonia in the right lung of the same animal. After 96 hours of sulfonamide chemotherapy Type I pneumococci were inoculated intrabronchially upon both lesions. The photomicrographs in Fig. 4 show that phagocytosis of the organisms was equally striking in the two lungs.

A sterile pneumonia was produced by the intrabronchial injection of mucin alone, and virulent pneumococci (6 hour culture) were then inoculated onto this resolving lesion. As illustrated in Fig. 5, there was observed the same active phagocytosis of pneumococci even though the animals had had no previous contact with the pneumococcus.[3] The possibility that a species-specific pneumococcal antibody is involved in the phagocytosis would, therefore, appear to be remote.

Since type-specific antibody is the only immune body known to opsonize virulent encapsulated pneumococci, the following experiment was performed as a final test of the possibility that type-specific opsonins are the cause of the

[3] Because these rats did not receive sulfapyridine the experiment adds additional support to the conclusion that the phagocytic reaction in the lung is in no way dependent upon the action of the drug.

phagocytic reaction. Rats recovering from Type II pneumonia (96 hours of chemotherapy) were given an intravenous injection of an excessively large amount of Type I polysaccharide (10 mg.) just before the intrabronchial inoculation of Type I pneumococci upon the resolving lesions. The inoculated Type I pneumococci were suspended in a high concentration of Type I polysaccharide (15 mg. per cc.). Inasmuch as pneumococcus polysaccharide is known to combine with and inactivate homologous type-specific antibody (11), the presence of phagocytosis in the lungs of rats so treated with polysaccharide (Fig. 6) is strong evidence against the hypothesis that type-specific opsonins are responsible for the phagocytosis observed in the resolving pneumonic lesion.

From these observations it is concluded that the phagocytosis of pneumococci in the lungs is unrelated to the presence of type-specific antibody in the pneumonic lesion.

DISCUSSION

Early studies on the curative action of sulfapyridine upon pneumococcal infections led to the conclusion that the drug exerts a direct action upon the capsule of the organism. From a study of experimental pneumococcal peritonitis in mice, Whitby (12) concluded that "the capsules became swollen and crenated and eventually disappeared" as the result of sulfapyridine therapy. This conclusion was in accord with the theory of Levaditi and Vaisman (13), who suggested that sulfonamide drugs also prevented the capsulation of hemolytic streptococci. In accordance with this theory it might be concluded that the phagocytosis of pneumococci observed in the lungs of pneumonic rats treated with sulfapyridine was due to a direct action of the drug upon the capsule of the pneumococcus. However, Whitby's early conclusions were not confirmed by subsequent studies (14). The experiments reported in the present paper also fail to confirm the view that sulfapyridine exerts an opsonizing effect upon the pneumococcus. Growth of the organism in high concentrations of sulfapyridine in no way altered its resistance to phagocytosis. Hence it can be concluded that the phagocytic reaction that occurs in the lungs of animals recovering from experimental pneumonia is due to some factor other than injury to the pneumococcus capsule by the drug.

The sera of most adult human beings (4, 15) as well as those of several laboratory animals (16–19) are known to contain natural antibodies against many different types of pneumococci. It has been suggested that natural circulating opsonins play an important rôle in the mechanism of recovery from pneumococcal pneumonia and that they may even explain the phagocytic reaction that occurs in the lung (5). Some doubt is cast upon this suggestion

however, by the fact that natural antipneumococcal immune bodies are known to disappear from the serum of many patients during the active stage of pneumonia and may be absent at the time of recovery (20). Conclusive disproof of the hypothesis is offered by the present experiments in which the animal selected for study was found to possess no natural antibody against the pneumococcus used. Though the rat possesses no natural antipneumococcal opsonins, its lung has been shown to be the seat of an active phagocytic reaction which readily destroys the offending bacteria.

The effective phagocytosis might likewise be explained by the presence of acquired antibody in the blood serum. There is clinical evidence, however that patients recovering from pneumonia, either spontaneously or after sulfonamide chemotherapy, not infrequently experience a crisis in the disease several days before immune bodies appear in the serum (21–25). This evidence alone is inconclusive, for it is conceivable that the crisis might occur before appreciable phagocytosis took place in the lungs. The present experiments were designed to determine the relationship between the onset of phagocytosis and the appearance of antibodies in the serum. It is clear that phagocytosis begins in the lung at least 48 hours before immune bodies can be detected in the serum. The phagocytosis observed in the lungs of rats recovering from experimental pneumonia cannot, therefore, be accounted for by the presence of either natural or acquired antibodies in the serum.

Although antibody cannot be demonstrated in the serum of rats when they first exhibit the pulmonary phagocytic reaction, it is still possible that antibody may be present in the local pulmonary lesion. Robertson (26) observed phagocytosis in the lungs of dogs dying of experimental pneumonia, and since no antibody could be demonstrated in the serum of these animals, he suggested that immune bodies may be produced locally in the lungs and thus cause phagocytosis without being present in detectable amounts in the circulating blood. It has been demonstrated also that recently immunized experimental animals exhibit an increased resistance to pneumococcal infection several days before immune bodies can be demonstrated in their serum (10). There is considerable doubt, however, as to whether significant amounts of type-specific antibody can exist in an unbound state in the pneumonic lung. Homologous type-specific polysaccharide is known to combine with specific antibody and to nullify its effectiveness as an opsonin (10). The lungs of patients dying of lobar pneumonia have been shown to contain huge quantities of capsular polysaccharide (27), and specific carbohydrate has likewise been demonstrated in the lungs of dogs recovering from experimental pneumonia as long as 2 months after recovery (28). Even if it is assumed that antibody accumulates locally in the pneumonic lesion, it apparently does not neutralize all of the

antiphagocytic polysaccharide and therefore cannot be expected to opsonize the pneumococci.

The present observations all offer strong evidence against the hypothesis that the phagocytosis is due to the local accumulation of type-specific opsonins in the pneumonic lesion.

SUMMARY

The phagocytosis which occurs in the lungs of rats receiving sulfonamide is due neither to an opsonizing action of the sulfonamides nor to type-specific antibody. The evidence presented indicates that the destruction of the pneumococci is brought about by a phagocytic mechanism independent of both opsonization and capsular injury.

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EXPLANATION OF PLATES

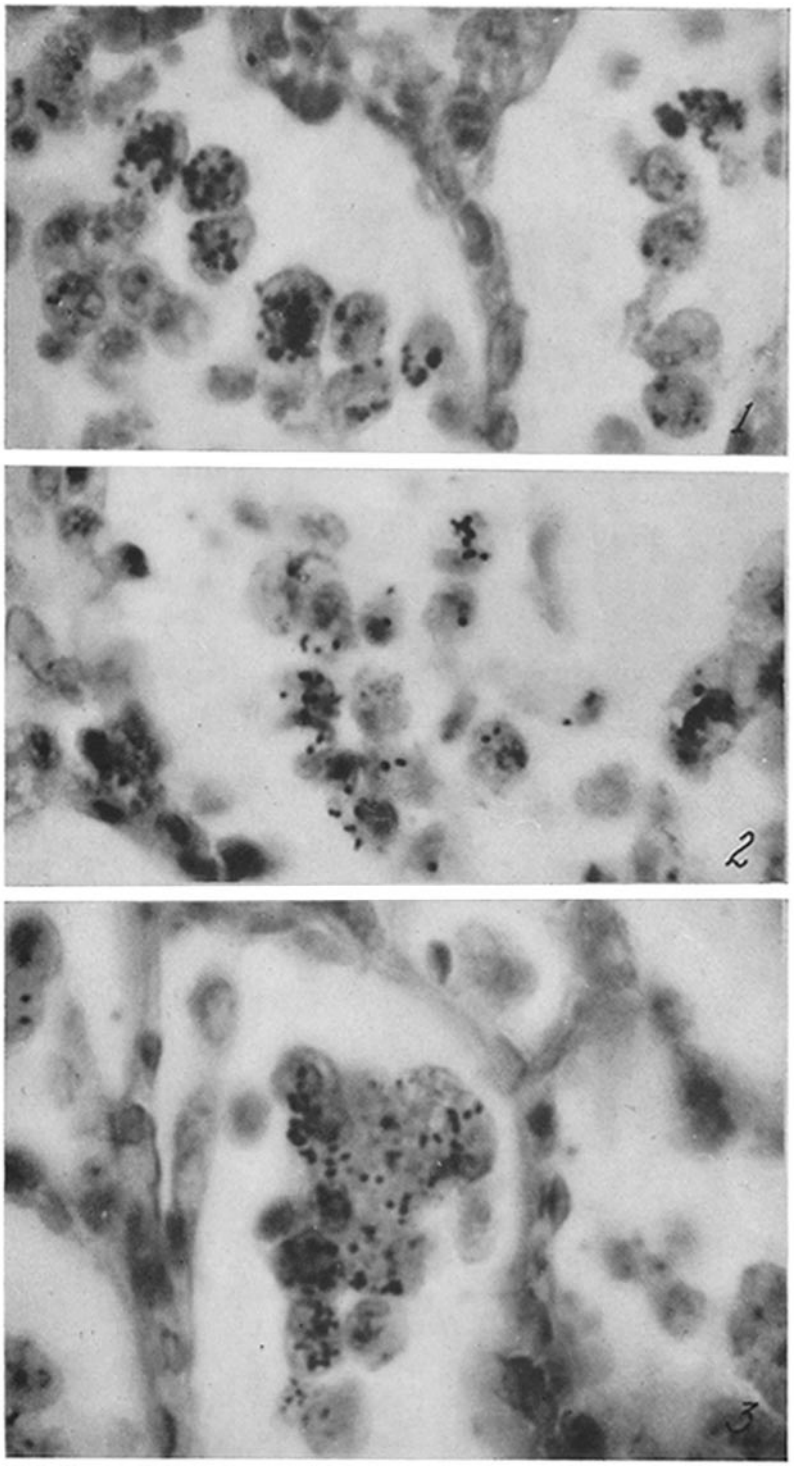
Sections stained by the Gram-Weigert technique.

PLATE 15

FIG. 1. Phagocytosis of virulent Type I pneumococci (6 hour culture) inoculated onto resolving pneumonic lesion of homologous type. Second inoculum injected intrabronchially after 72 hours of sulfonamide treatment, at which time none of the pneumococci from the original inoculum was still visible (1). Animal sacrificed 1 hour after second inoculation. × 945.

FIG. 2. Same as Fig. 1, 4 hours after second inoculation. × 945.

FIG. 3. Phagocytosis of virulent Type II pneumococci (6 hour culture) inoculated onto resolving pneumonic lesion of *heterologous* type (Type I). Second inoculum injected after 96 hours of sulfonamide treatment. Animal sacrificed 4 hours after second inoculation. × 945.



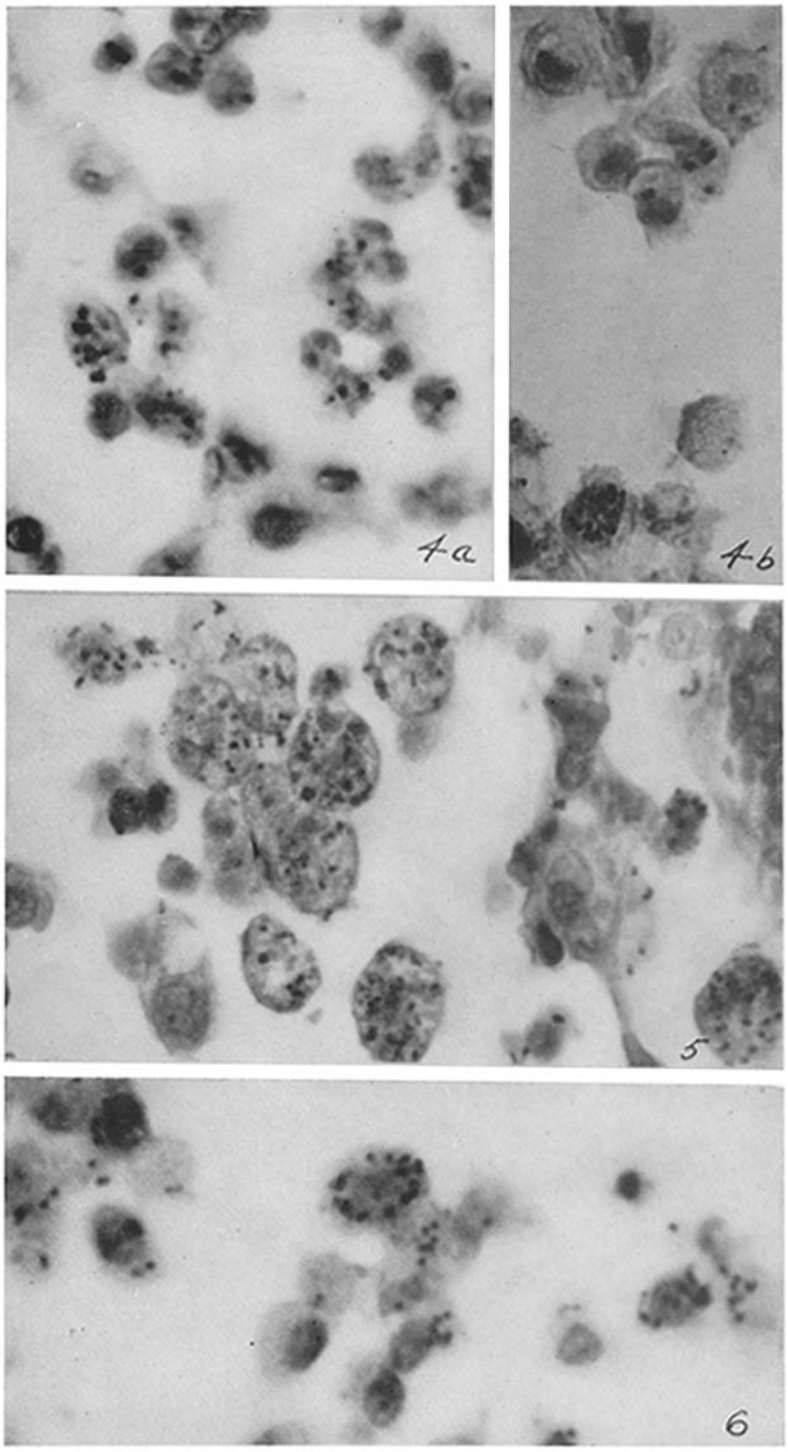
(Wood *et al.*: Mechanism of recovery in pneumococcal pneumonia. III)

PLATE 16

FIG. 4. Type I pneumonia was produced in the left lung and Type II pneumonia in the right lung of the same animal. After 96 hours of sulfonamide chemotherapy (when all of the original pneumococci had been destroyed) Type I pneumococci (6 hour culture) were inoculated intrabronchially upon both lesions. Photomicrographs 4 *a* (left lung) and 4 *b* (right lung) show that phagocytosis was equally striking in the two lungs. × 945.

FIG. 5. Sterile pneumonia was produced by intrabronchial injection of mucin alone, and virulent Type I pneumococci (6 hours culture) were inoculated onto the resolving lesion 4 days later. The phagocytic reaction was active 4 hours after inoculation of pneumococci, although animals had had no previous known contact with pneumococci and received no sulfonamide treatment. × 945.

FIG. 6. Rats recovering from Type II pneumonia (96 hours of sulfonamide chemotherapy) were given an intravenous injection of Type I polysaccharide (10 mg.) just before the intrabronchial inoculation of Type I pneumococci upon the resolving lesion. The Type I pneumococci were suspended in a high concentration of Type I polysaccharide (50 mg. per cc). Phagocytosis was striking 4 hours after the second inoculum in spite of the fact that pneumococcal polysaccharide is known to combine with and inactivate homologous type-specific opsonins. × 945.



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