THE ROLE OF THE ALVEOLAR MACROPHAGE IN THE CLEARANCE OF BACTERIA FROM THE LUNG*

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PLATES 6 AND 7

Inhaled bacteria disappear rapidly from the lungs of experimental animals (1–3). This early clearance of bacteria is thought to be an important process in non-specific resistance to infection in the bronchopulmonary tree. Quantitative methods of study have provided a sensitive tool for detecting small as well as large changes in the efficiency of these mechanisms of resistance. A wide variety of chemical, hormonal, and environmental agents depress, to different degrees, the rate at which inhaled cultivable bacteria disappear from the lungs (4). Such studies support epidemiologic evidence that multiple agents may be involved in the pathogenesis of chronic infections

It is not clear to what extent each of the several component defense systems of the bronchopulmonary tree participates in the initial inactivation of inhaled bacteria. Although the mucociliary stream is frequently credited with this cleansing action, indirect evidence suggests that bacterial clearance may be accomplished by alveolar macrophages. In an attempt to answer this question directly, the pathway through the lung followed by inhaled bacteria was first traced by bacterial localization studies using conventional histologic and immunofluorescence methods. The latter method was used to localize bacterial antigen where the structural integrity of the organism had been destroyed. Then, radio-labeled viable bacteria were used to compare the rate at which inhaled bacteria lose their viability with the rate at which they are physically removed from lung tissue. In this way the action of bactericidal mechanisms such as phagocytosis was compared with the action of removal mechanisms such as the mucociliary stream and lymphatic drainage. The results of both studies point to the phagocytic action of macrophages as the major mechanism of early resistance to bacterial infection.

Methods

Animal Infection.—White Swiss mice of the Charles River breed, weighing 20 to 25 gm, were exposed in a closed vented chamber to bacterial aerosols of either *Staphylococcus aureus* (coagulase-positive, FDA 209 P phage type 42D) or *Proteus mirabilis* (laboratory strain). The bacterial suspensions used in producing the aerosols were prepared from overnight broth cultures incubated at 37°C in a shaker water bath. Aliquots of the broth cultures were centrifuged at 6000 RPM for 30 minutes. The sedimented bacteria were resuspended in M/15 phosphate buffer of pH 7.4 to give a final concentration of 2 to 5 $\times$ 10^9 bacteria/ml. Five to 8 ml of the bacteria suspension were then placed in the nebulizer for infection of the animals.

The aerosol exposure apparatus used in these experiments was constructed by Laurenzi and associates (3) as a small inexpensive model of similar devices used by Lurie (5) and by Middlebrook (6) in quantitative studies of tuberculous infection in laboratory animals. The bacterial aerosol is generated in a nebulizer by compressed air flowing at 5 to 6 liters per minute. This aerosol, consisting largely of particles less than 3 μ in diameter, is directed into the open end of the mixing chamber where the droplets are dried and dispersed by a large volume of secondary air drawn into the mixing area by a vacuum pump at the distal end of the apparatus. The bacterial cloud is then drawn into the animal exposure chamber, in which the animals are placed individually wrapped in rubber mesh bags. The chamber is then exhausted through a series of bacterial filters. Previous studies have shown that the method permits quantitative infection of the lungs of small animals, and that the results are predictable and reproducible.

Histologic Studies.—The initial histologic studies were performed on animals exposed to the aerosol for 30 minutes, but because the numbers of bacteria deposited were relatively small for this type of study, later studies were performed on animals exposed continuously for 4 hours. In the experiments in which 4-hour exposures were done, it was necessary to shut down the aerosol for 1 to 2 minutes on three or four occasions during the exposure in order to refill the nebulizer. Immediately after exposure to the bacterial aerosol, the animals were killed and the lungs removed for histologic study. Parallel animals were killed for quantitative culture of the lungs. Initial histologic studies were done using the Macallum-Goodpasture tissue Gram stain. However, since this method fails to stain non-viable bacteria reliably, most of the studies were made using immunofluorescence methods.

Preparation of Antisera and Conjugates.—The specific antiserum against *Staphylococcus aureus* was prepared in albino rabbits by injecting on 3 successive days each week heat-killed organisms from an overnight broth culture, doubling the dose each week from a dose of 0.1 to 1.0 cc. Then, increasing doses (0.1 to 1.0 cc) of live organisms from an overnight broth culture were given on the same schedule. The animals were bled from the heart at the end of 6 weeks and the serum titrated against a saline suspension of live organisms.

The specific antiserum against *Proteus mirabilis* was prepared in albino rabbits using acetone-killed organisms. Intravenous injections were given in increasing doses 3 days weekly for 3 weeks. In the 4th week, three consecutive daily injections of a suspension of live organisms were given. One week later, the animal was bled from the heart, and the titer of the harvested serum determined.

The whole antisera were conjugated with fluorescein isothiocyanate and the gamma globulin fraction separated by diethylaminoethyl cellulose column chromatography according to the method of Riggs, Loh, and Eveland (7). In some of the staphylococcal studies and in all of the proteus studies, the fluor was absorbed with mouse liver powder just before use.

Preparation of Tissues.—The tissues containing staphylococci were frozen with isopentane-liquid nitrogen, because difficulty was encountered with preservation of the antigen using ethanol, formalin, or acetone fixatives. Histologic sections were cut in the cryostat, fixed for 15 minutes in acetone, rinsed in phosphate-buffered saline (PBS) of pH 7.2, and stained.

The tissues containing proteus organisms were fixed in 95 per cent ethanol at 4°C, according

to the method of Sainte-Marie (8), and embedded in paraffin. Before staining, the 2 to 4 μ sections were passed through xylene, graded concentrations of ethanol, and PBS.

For immunofluorescent staining, the slides were layered with conjugated antiserum for 60 minutes, washed in PBS, and mounted in buffered glycerol. In some of the staphylococcal studies the sections were counterstained with 4 per cent Evans blue according to techniques developed by Teplitz (9). Controls of specificity included inhibition of specific staining by previous treatment of the sections with homologous but not heterologous immune sera. The fluorescent slides were examined under ultraviolet light using a Zeiss photomicroscope or GFL microscope equipped with Osram HBO 200 ultraviolet light source. The photomicrographs were recorded on high speed ektachrome type B film and black and white prints were made from the color transparencies.

Radiotracer Studies.—The radio-labeled bacteria were prepared by growing the bacterial strain in a medium consisting of 25 ml 1 per cent casamino acid solution to which was added 2.0 mc carrier-free radioactive phosphorous (P^{32}) as sodium phosphate. Optimal uptake of the tracer and adequate growth were obtained when the phosphate was first precipitated from the casamino acid solution as magnesium ammonium phosphate and then standard beef heart infusion broth added to a final concentration of 1 per cent for *S. aureus*, and 2 per cent for *P. mirabilis*. The cultures were then centrifuged at 6500 RPM and washed twice with phosphate buffer at pH 7.4 to remove unincorporated P^{32}. The bacterial sediment was resuspended in 5 ml of buffer in the staphylococcus studies and 9 ml in the proteus studies, and placed in the nebulizer.

The animals were divided into groups of four at the end of a 30 minute exposure to isotope-labeled bacteria. One group was killed immediately, a second group was killed in 2 hours, and a third in 4 hours. The lungs of each mouse were removed by transection of the mainstem bronchi and homogenized in a glass tube grinder. An aliquot of each homogenate was quantitatively cultured by pour plate technique and a second aliquot, appropriately diluted, was used for counting radioactivity in a beta counter.

In calculating results, the radioactive count of each specimen was corrected for background and dilution and expressed as counts per hour per animal's lungs. The results of quantitative culture were expressed as the number of bacteria per animal's lungs. The mean of each group of animals at each time period was calculated. Bacterial clearance was then expressed as the number present immediately after exposure, minus the number present at time t, divided by the initial number of bacteria. The statistical methods reported elsewhere (4) were used in calculating the mean and standard error of the ratios expressing clearance.

Testing of both the sedimented bacteria and the supernatant for content of P^{32} showed that approximately 90 per cent of the total radioactivity of the aerosol was contained in the bacterial sediment. Thus, up to 10 per cent of the initially deposited radioactivity may be due to unincorporated P^{32}, which would be expected to diffuse out of the lung rapidly.

RESULTS

Animals exposed to the bacterial aerosol for 30 minutes have approximately 40,000 viable organisms in the lungs at the conclusion of the exposure period. These numbers can be increased approximately tenfold by exposing the animals continuously for 4 hours. Even at this level, only a very few organisms are visible per section of lung tissue examined. In addition to increasing the total number of organisms, the 4 hour exposure allows time for the clearing mechanisms to operate, and thus to find organisms at various stages of the clearing process.

In the animals killed after 3 minutes' exposure to the bacterial aerosol, the Gram-stained lung sections showed very few cocci scattered in the section lying either free in the alveolar spaces or within alveolar cells (Figs. 1 and 2). In immunospecifically stained sections, fluorescent material was seen to lie principally at the alveolar level of the bronchopulmonary tree. Both the staphylococci and the proteus organisms could be seen lying singly or in pairs in respiratory bronchioles or alveoli and scattered sparsely through the section. Most of the organisms, however, were seen to lie within alveolar cells which were lining or closely adjacent to the alveolar septa. While the proteus organ-

TABLE I

Deposition and Clearance of P^{32}-Labeled Staphylococcus aureus

Hrs. after infection	No. of animals	Bacterial count in lungs (mean ± se)	P^{32} counts/hr. in lungs (mean ± se)
0	8	13,300 ± 950	13,800 ± 800
2	8	2490 ± 350	11,100 ± 810
4	8	1160 ± 300	11,100 ± 920

TABLE II

Deposition and Clearance of P^{32}-Labeled Proteus mirabilis

Hrs. after infection	No. of animals	Bacterial count in lungs (mean ± se)	P32 counts/hr. in lungs (mean ± se)
0	16	32,900 ± 4000	10,600 ± 830
2	12	17,400 ± 1900	11,100 ± 860
4	12	7700 ± 750	9150 ± 810

isms could be seen to maintain their rod shape within these phagocytic cells, the staphylococcal material seemed more diffusely spread in the cytoplasm of these cells (Fig. 3). Occasional bacteria could be seen lying free on the mucosa of bronchi containing continuous epithelium, but there were relatively few at this level of the bronchial tree.

In the lungs of the animals exposed to an aerosol of proteus for 4 hours, there were organisms in the respiratory bronchioles and alveoli (Fig. 4). In addition large mononuclear phagocytes containing variable numbers of bacteria per cell could be seen lying on the ciliated mucosa of the larger bronchi (Fig. 5). The point of origin of these bacteria, whether bronchial or alveolar, could not be determined. The two most striking features of these preparations were first, that relatively few free bacteria were seen at the level of bronchi lined with ciliated columnar epithelium, and secondly, that relatively few bacteria were seen to be free of phagocytosis in the lung. Organisms were not seen in the peri-

vascular or peribronchial lymphatic vessels. It is possible that at this relatively low inoculum, lymphatic drainage plays only a negligible role.

Routine hematoxylin and eosin–stained sections prepared from the tissue blocks showed no recognizable pathologic changes. Polymorphonuclear leuko-
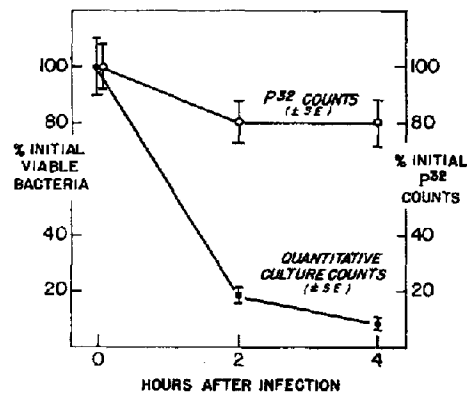
TEXT-FIG. 1. Clearance of P^{32}-labeled *S. aureus* from mouse lung. The change in numbers of viable organisms is compared with the change in radioactivity of the lung.
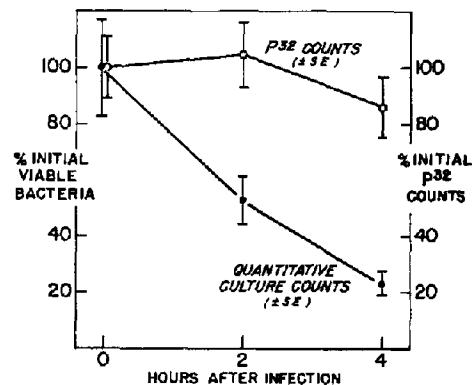
TEXT-FIG. 2. Clearance of P^{32}-labeled *P. mirabilis* from mouse lung. Measurements as in Text-fig. 1 with *P. mirabilis* as the test organism.

cytes do not appear to participate in the removal of a low inoculum of bacteria inhaled as droplet nuclei.

The data of the radiotracer studies are summarized in Tables I and II. In the staphylococcal studies the initial P^{32} count was virtually identical with the count of viable bacteria. Over the subsequent 4 hours, the viable count rapidly declined, while the radioactive count remained virtually stable. The results of the proteus studies are similar. The higher initial viable count of the proteus is

due to a greater concentration of organisms in the aerosol suspension. The lower specific activity of the proteus may be due to the higher concentration of added broth necessitated on an empirical basis to attain adequate growth. The slow rate of killing of the proteus presumably indicates a greater resistance of this organism to the clearance mechanisms. This difference will be examined in greater detail in another report.

The viable and radiotracer clearance curves are plotted in Text-figs. 1 and 2 for comparison. A marked disparity is seen between the rates of decline in viable bacteria and in radioactive counts. By culture techniques, the staphylococci were reduced in number by 81 per cent in 2 hours and 91 per cent in 4 hours, and the proteus were reduced by 47 per cent in 2 hours and 67 per cent in 4 hours. Over the same time period, radioactivity declined only 14 per cent for proteus and 20 per cent for staphylococcus. The decline in numbers of viable bacteria occurs at a very different rate from that of physical removal of bacteria from the lung.

DISCUSSION

Although cultural methods measure the total antibacterial action of the lung, and histologic methods permit localization of inhaled bacteria, neither of these methods is suitable for quantitating the relative action of the mucociliary stream, lymphatic drainage, and alveolar phagocytosis. This information is needed to understand the mechanisms of bacterial clearance and to explain the inhibiting effects that a variety of agents have on bacterial clearance. In terms of ridding the lung of viable bacteria, the clearing mechanisms may be divided into two categories: (a) mechanisms which physically remove bacterial material from the lung and (b) mechanisms which destroy the viability of the organism, but which may or may not move the bacterial matter from the lung. The mucociliary apparatus and lymphatic drainage system operate in the first category, while phagocytic mechanisms are in the second. Although the direct visualization of bacteria by fluorescent means indicated that pulmonary macrophages, and not the mucociliary stream, seemed to account for most of the antibacterial activity, direct proof was obtained by the studies of the radioactive bacteria. Those latter experiments show that the rapid disappearance of inhaled viable bacteria is not due to mechanical removal. Only if viable bacteria are transported more rapidly than non-viable bacteria by the mucociliary stream could the more rapid decline of viability be attributed to mechanical removal mechanisms. Such a possibility seems unlikely. The persistence of labeled bacterial matter, while viability rapidly declines, indicates that the major part of bacterial clearance is *in situ* bactericidal activity. Whereas surface enzymatic or antibody activity may play a role in this bactericidal action, the fluorescence studies show that the inhaled bacteria are rapidly taken up by phagocytic

cells on the surface of the alveoli and respiratory bronchioles. These studies implicate the alveolar macrophage in the bactericidal action of the lung.

The appearance of bacteria in the bronchioles and alveoli indicate that organisms inhaled as droplet nuclei penetrate the filtering actions of the upper respiratory tract and appear to follow the deposition pattern of dust particles of similar size (10). It can be assumed that the lung is continuously exposed to variable numbers of bacteria as well as to dust that is suspended in inhaled air. The normal sterility of the distal portions of the lung and the rapid decline in viable bacteria after infection with the aerosol method attests to the effectiveness of the pulmonary defense mechanisms.

The mechanisms responsible for the clearance from the lung of low levels of inhaled material appear to be distinct from the more intense inflammatory response which occurs in active, invasive bacterial disease. Whereas the latter is dominated by exudation of edema fluid and polymorphonuclear leukocytes, the former appears to be characterized by the phagocytic activity of the mononuclear macrophages in the alveoli and respiratory bronchioles (11, 12). Although phagocytic ingestion appears to be the first step in the removal of bacteria deposited in the respiratory bronchioles and alveoli, the bacteria must then either be killed or eliminated to prevent later multiplication and disease. The radioactive studies show that killing precedes removal. The appearance of diffuse staphylococcal antigen in the macrophages suggests intracellular dissolution and destruction of the organisms.

The appearance of proteus-containing macrophages on the surface of the bronchial mucosa, may indicate either that these bacteria were deposited and phagocyted at that site, or that the macrophages, after phagocyting the bacteria in the alveoli, slowly migrated to the mucociliary stream and were then eliminated. The absence of the phagocytic cells in the bronchi of animals exposed for only 30 minutes, supports the latter hypothesis. These observations suggest that bacterial clearing depends upon the phagocytic activity of the pulmonary macrophage, whereas ultimate removal of bacterial products depends upon transport of the macrophage with its contents of bacterial products along the mucociliary stream. Clearance, in the sense of killing plus removal, would thus depend upon the integrated function of both the alveolar macrophage and the mucociliary stream.

The difference between the clearance rates of radioactivity and the disappearance of viability points out the major and important distinction between the behavior of the lung toward inhaled dust particles and toward inhaled bacteria. Since the pathogenesis of infectious disease depends, in general, on the presence of viable organisms, the efficacy of pulmonary defense mechanisms must be measured in these terms. It can be expected that the ability to kill an organism depends on factors quite distinct from the ability to move particu-

late material mechanically. In this regard, the impairment of clearing caused by a variety of chemical, hormonal, and environmental agents (4) can be reasonably attributed to impairment of the bactericidal action of the macrophages, either through depression of phagocytic uptake, or by inhibition of intracellular digestion.

SUMMARY

Pulmonary clearance of bacteria was studied using histologic, bacteriologic, and radiotracer methods. When mice were exposed to an aerosol of P^{32}-tagged *Staphylococcus aureus* or *Proteus mirabilis*, and the rate of disappearance of viable bacteria compared with the rate of their mechanical removal, it was found that bacterial viability declined 80 to 90 per cent in 4 hours, whereas radioactivity declined by only 14 to 20 per cent. The marked disparity in these rates indicated that mechanical removal comprised a relatively small fraction of the total clearing process. The *in situ* bactericidal action of the lung predominated over the mechanical removal process in achieving clearance of the inhaled bacteria. By immunofluorescent methods, the inhaled bacteria were found to be localized in the alveolar spaces and within alveolar macrophages. These observations suggest that the bactericidal action of the bronchopulmonary tree is due primarily to the phagocytic activity of the alveolar macrophages, and that the action of the mucociliary stream, in relation to bacterial particles, may be largely related to the transport from the lung of phagocytes containing material of bacterial origin.

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EXPLANATION OF PLATES

PLATE 6

Fig. 1. Section of mouse lung prepared immediately after 30 minute exposure of the animal to aerosol of *S. aureus*. Staphylococci are seen within the cytoplasm of a mononuclear macrophage in the alveolar tissue. Macallum-Goodpasture stain. × 2500.

Fig. 2. Section of mouse lung prepared immediately after 30 minute exposure of the animal to aerosol of *S. aureus*. Portions of three alveoli are seen separated by alveolar septal tissue. The staphylococci have been ingested by what appears to be an alveolar septal cell. Macallum-Goodpasture stain. × 2500.
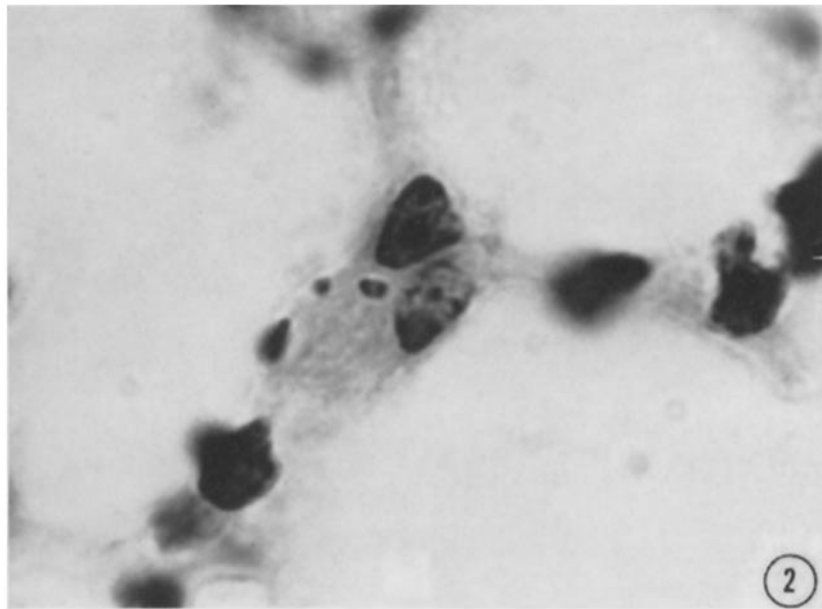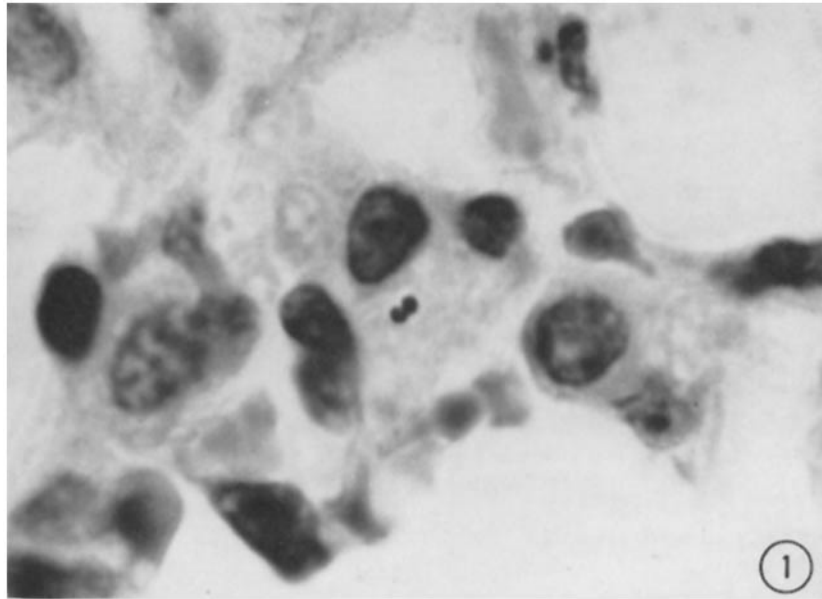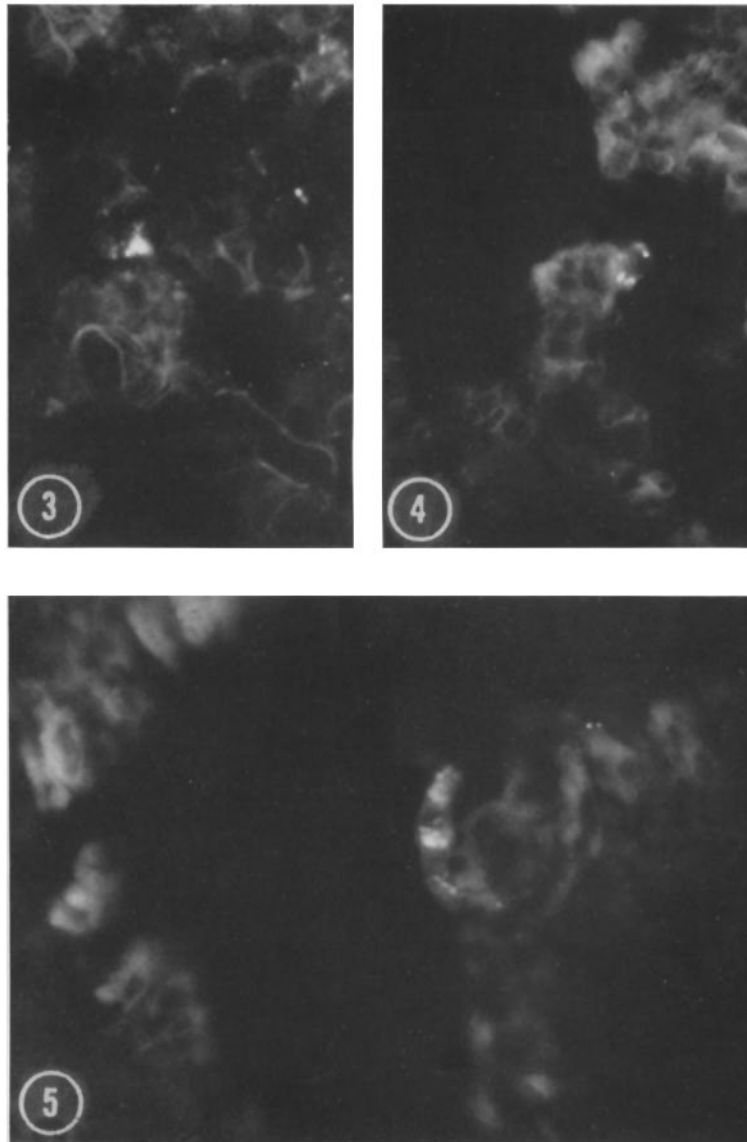
(Green and Kass: Alveolar macrophage)

PLATE 7

FIG. 3. Section of mouse lung after exposure to *S. aureus* aerosol for 30 minutes. A clump of specifically stained staphylococcal antigen is seen in a mononuclear cell of the alveolar tissue. A single coccus is visible to the left. Stained by fluorescein conjugated anti–*S. aureus* gamma globulin. Approximately × 430.

FIG. 4. Section of mouse lung after exposure to *P. mirabilis* aerosol for 4 hours. The fluorescent bacilli are present in the cytoplasm of a mononuclear macrophage located in a respiratory bronchiole. Stained by fluorescein-conjugated anti–*P. mirabilis* gamma globulin. Approximately × 430.

FIG. 5. Section of mouse lung after exposure to *P. mirabilis* aerosol for 4 hours. The mononuclear macrophage lying on the bronchial mucosa contains clumps of fluorescent bacteria. Stained by fluorescein-conjugated anti *P. mirabilis* gamma globulin. Approximately × 430.

(Green and Kass: Alveolar macrophage)