Pneumonic Plague in Monkeys

An Electron Microscopic Study

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AIRBORNE PLAGUE is a highly contagious infection having an abrupt onset and rapidly fatal course, if untreated.[1] At necropsy the lungs display necrotizing pneumonia. Early histopathologic alterations consist of extensive intra-alveolar edema in which myriads of *Pasteurella pestis* are found, with minimal leukocytic reaction or detectable damage to the tissue.[1] Infected edema fluid is also found at the margins of consolidated areas and is the initial abnormality in secondary lesions.[1] Other bacterial pneumonias, such as pneumococcal, seem to have a similar pathogenesis, as studied in the rare human cases with abbreviated disease due to intervening accident[2] or in experimental animals,[3] but differ from plague in the more rapid addition of leukocytes, fibrin, and red blood cells to the exudate.

The electron microscope has been successfully employed in the study of a variety of inflammatory reactions and their components, such as leukocyte migration,[4] phagocytosis,[5,6] and vascular permeability,[7] but there have been few reports of the ultrastructural manifestations of bacterial pneumonia.[8-10] In view of the value of electron microscopy in detecting tissue damage in experimental pulmonary edema prior to the appearance of lesions visible with the light microscope,[11,12] it seemed appropriate to examine the lungs of animals exposed to aerosols of *P. pestis* in order to describe the ultrastructural changes in necrotizing pneumonia and, hopefully, to determine the pathogenesis of the extensive, early edema.

Materials and Methods

Animals

Rhesus monkeys (*Macaca mulatta*), imported from India, were housed locally for at least 90 days before use. Young adults of both sexes weighing between 2.6

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In conducting the research reported herein, the investigator adhered to Guide for Laboratory Animal Facilities and Care established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, NAS-NRC.

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and 3.25 kg. were housed in pairs and were fed Purina monkey chow (Ralston Purina Co., St. Louis, Mo.) and water ad libitum. Tuberculin testing was performed monthly and only negative reactors were used.

Organism and Exposures

The KIM-10 strain of *P. pestis* was used. The isolation, maintenance and cultural characteristics have been described,[13] as have the methods of aerosol generation, animal exposure, and dosimetry. The calculated inhaled dose in these experiments averaged 1360 bacilli per monkey.

Sampling

Pairs of animals were chosen at random at 6, 12, 24, 48, 72, and 96 hr. after exposure. Each monkey was given an intramuscular injection of Sernylan (Parke, Davis & Company, Detroit, Mich.) in a dosage of 2 mg./kg. body weight, which produced stupor within 10 to 15 min. One of each pair then was given an intravenous injection of colloidal carbon (Pelikan C 11/1431a, Gunther-Wagner Co., Hanover, Germany), 0.1 gm./100 gm. body weight. Three to five minutes later the animals were sacrificed with intravenous pentobarbital.

Tissue Preparation

Immediately after the pentobarbital injection, the chest was opened and the lungs were excised. They were dissected free of mediastinal tissues and weighed. They were fixed by endotracheal perfusion of cold 2% glutaraldehyde buffered to pH 7.2 by 0.1 M phosphate to give a total osmolality of 444 mosm. The perfused lungs were immersed in the same fluid at 0–4° C. for 1 hr., then examined grossly and sectioned. Five to ten 1-mm. cubes of tissue from representative areas of each lung were taken for electron microscopic examination, and sections of all lobes measuring 2 by 1 by 0.4 cm. were excised for paraffin embedding and light microscopy. The latter sections were 5–7 μ thick and were stained with hematoxylin and eosin and with Giemsa's stain. Processing for electron microscopy consisted of washing in phosphate buffer adjusted to 444 mosm. with 0.25 M sucrose, mincing in buffer, fixing in 1% osmium tetroxide for 1 hr., dehydrating in ethyl alcohol, embedding in Epon 812, sectioning at 400–800 Å with a Porter-Blum MT 1 microtome, and treating sections on unsupported copper grids with a saturated solution of uranyl acetate. Sections were examined and photographed in a RCA EMU 3-G microscope.

Results

Until approximately 60 hr. after exposure, there were no clinical signs of infection, so the 8 animals sacrificed prior to 72 hr. appeared healthy. Their lungs were grossly and microscopically normal. The remaining 4 animals developed temperatures of 104–105.3° F. between 60 and 72 hr. and had slight tachypnea (respiratory rate of 60–70/min.) They were otherwise clinically well, with no lethargy or loss of appetite. This illness is similar to that seen in previous studies of pneumonic plague in monkeys.[14] At necropsy, the proportion of lung weight to body weight of one of the 96-hr. animals far exceeded the average normal value (14.5 vs. 8.0), and that of the other 96-hr. animal was slightly

greater (9.7 vs. 8.0). Each of the 4 animals sacrificed at 72 and 96 hr. had pneumonia, the most extensive consisting of confluent gray hepatization of the right lower lobe in one animal at 72 hr. There was central liquefactive necrosis of this area, and the overlying pleura was dull and opaque with hemorrhagic foci. An area of increased consistency and excess fluid was present in the left upper lobe of this monkey, and similar foci, compatible with early lobular pneumonia, were found in the other 3 animals.

Light Microscopy

Microscopically, each of the 4 monkeys examined at 72 and 96 hr. had pneumonia, with a range of lesions in each animal that conformed to the many earlier descriptions of the disease in both man and animals.[1,15] The mildest infection was found in one animal at 96 hr., and consisted of extensive alveolar edema containing numerous bacilli, and a variable leukocytic infiltrate of the septums and alveoli (Fig. 1 and 2). In some areas, there were very few polymorphonuclear neutrophils within septal capillaries, but elsewhere individual alveoli were filled with the same cells. No changes in the alveolar walls could be detected initially, but after examining the electron micrographs it was possible to recognize subepithelial blebs in the areas of edema with the light microscope (Fig. 3). They were rounded elevations of the alveolar wall, 7–12 μ in diameter, containing eosinophilic fluid. Their nature could be ascertained only with the electron microscope (see below). Perivascular and peribronchial lymphatics in the vicinity of the early lesions were distended with fluid (Fig. 1). Bronchi and terminal bronchioles were not remarkable. The same histologic appearance characterized the secondary lesions.

More advanced lobular pneumonia consisted of alveolar consolidation by polymorphonuclear neutrophils and a few mononuclear cells; extensive infiltration of septums, and of interlobular, peribronchial, and perivascular connective tissue by the same cells; massive growth of bacilli in the alveoli and lymphatics; and foci of inflammation and necrosis of bronchiolar epithelium. Hemorrhages and focal necrosis of the alveolar septums were numerous in the centers of the confluent areas of pneumonia (Fig. 4).

The carbon injections did not reveal the site of increased vascular permeability. Prior to 72 hr. the only evidence of the presence of carbon consisted of diffuse black stippling of the blood in large vessels. In 2 animals with pneumonia, discrete aggregates of carbon occluded

arterioles and capillaries, both in the areas of pneumonia and elsewhere. No carbon was detected in the alveolar edema fluid or in lymphatics.

Electron Microscopy

With the aid of routine histologic sections and toluidine blue-stained, 1-μ-thick sections of each capsule of plastic-embedded tissue, it was possible to select for study with the electron microscope areas of the lung that represented a progression from minimal to maximal alterations of morphology. The progression is assumed to reflect the temporal sequence of inflammation, and the observations will be presented according to that interpretation.

No ultrastructural alterations were observed prior to 72 hr. Numerous sections from the 6-hr. and 48-hr. monkeys were examined with special diligence in an effort to locate *P. pestis* in the periods soon after exposure to the aerosol and just preceding the onset of frank pneumonitis. Although the sections included portions of distal bronchi, bronchioles, alveoli, venules, veins, and lymphatics, neither organisms nor changes in the tissues were observed.

Edema. Sections from the areas of alveolar edema found with the light microscope showed numerous plague bacilli in the alveoli but none in the tissue. There were many areas with no morphologic changes despite the presence of the organisms (Fig. 5). Fluid in the alveoli was not sufficiently dense to be recognized. Interstitial edema could be detected by its effects in separating normal structure and was found in the absence of other tissue alterations. Perivascular and peribronchial connective tissues were widened by finely granular material that ranged in opacity from that of background to that of plasma. Interstitial fluid also accumulated beneath the thin segments of the alveolar epithelium (Type I cell), resulting in the large blebs or blisters that were visible with the light microscope. In some sections it was impossible to determine whether the fluid was extracellular or within a large intracellular vacuole (Fig. 6). However, other sections clearly revealed continuity of the fluid with the interstitial space (Fig. 7). Although affected alveolar epithelial cells were strikingly attentuated by the blisters, they were generally not otherwise remarkable. A few cells showed small cytoplasmic vacuoles in the cytoplasm near the blebs. Some blebs in the areas having a cellular infiltrate also contained leukocytes or erythrocytes; however, carbon was rarely found in the blebs, even when present in nearby vessels. As the pneumonia progressed, interstitial edema became more extensive and spread from the peritruncal areas to the thin portions of the septums. In these areas blebs were no longer seen.

Endothelial Lesions. The capillary endothelium showed minimal, focal cytoplasmic alterations in the areas of edema. Swelling of the cells with rarefaction of the cytoplasmic matrix was seen rarely in the early stages but became common in the more inflamed areas. This change often was restricted to part of a single cell, leaving organelles and pinocytotic vesicles intact, and was therefore considered to be a *degenerative* phenomenon. Other early endothelial changes consisted of the formation of multivesicular bodies, lipid vacuoles, and cytoplasmic inclusions of indeterminate nature. The appearance of membrane-like structures, or myelin figures, within the cytoplasm may indicate degeneration of some of the internal membranes of the cell. No increase in the number or size of the pinocytotic vesicles was noted at any stage.

More advanced endothelial damage was found in the areas of *necrosis*, where all elements of the tissue were involved. These changes consisted of swelling of cells, opacification of the cytoplasmic matrix, vacuolation of mitochondria, and disruption of cell membranes (Fig. 8). There were no intravascular thrombi at any time during this study, and even when small vessels were plugged with carbon, the occasional platelet in the area appeared intact and retained its granules. No fibrin was found.

Cellular Exudate. Extravasation of erythrocytes was noted in some areas with the light microscope (Fig. 1) and electron microscope. Very infrequently, an erythrocyte was observed leaving a capillary for the interstitial space. A gap in the endothelial lining was associated with alterations in the cytoplasm of the cells at the site, which appeared more dense and homogeneous than normal, with no vesicles. Otherwise the tissue in these areas appeared unaltered. No erythrocytes were found to emerge from veins or venules.

Leukocyte migration could be traced from capillaries and more often from venules into the interstitium and thence into the alveoli (Fig. 9 and 10). Polymorphonuclear neutrophils and monocytes appeared simultaneously within vessels, in the interstitium, and in the alveoli, although neutrophils outnumbered monocytes throughout the period of this study. Eosinophils were found in the more intensely inflamed and necrotic areas. Lymphocytes and plasma cells were not part of the reaction. The passage of leukocytes through the vessel wall was accompanied by either no change or minor alterations of the endothelium (Fig. 9), and the defects appeared to be between cells. Gaps permitting the migration of leukocytes out of vessels also allowed the egress of carbon (Fig. 9), which otherwise was confined to the blood plasma or to cytoplasmic vacuoles of monocytes. The cytoplasm of alveolar epithelial

cells at the sites of leukocytic migration was homogeneously granular and devoid of pinocytotic vesicles (Fig. 10), just as that of the endothelial cells.

Interactions of Leukocytes and Organisms. Neutrophils were plentiful in vessels, interstitium, and alveoli, and in the last site were close to many plague bacilli, yet phagocytosis by neutrophils was rarely observed. Some neutrophils in alveoli appeared degranulated, without the formation of vacuoles (Fig. 10), but serial sections to confirm these observations were not available. Monocytes did phagocytize *P. pestis,* as well as leukocytes (Fig. 11), erythrocytes, phospholipid profiles, and other debris. Lysosomes and phagocytic vacuoles were inconspicuous until the cells reached the interstitium or alveoli. The outcome of phagocytosis of the bacilli was generally unfavorable to the monocyte (Fig. 12), although occasionally a bacillus appeared to be undergoing digestion.

Epithelial Lesions. The alveolar epithelium displayed little early response to the presence of numerous *P. pestis* in the lumen (Fig. 5). Only in the areas of intense inflammation did the lining cells show morphologic abnormalities (Fig. 8). These consisted of focal swelling of the endoplasmic reticulum of both the granular (Type II) pneumocyte and the squamous (Type I) epithelial cell; mitochondrial swelling with loss of cristae; rarefaction of the cytoplasmic matrix; and, finally, sloughing of the cell, leaving exposed basement membrane.

Mast Cells. Normal-appearing mast cells were present in the adventitia of veins and venules early in the pneumonitis, when edema was the predominant feature. Later, when leukocytic exudation was active, some mast cells had empty granules. In the advanced lesions they joined in the general process of necrosis, with fragmentation of cytoplasm and disruption of the cell membrane.

Discussion

For approximately 60 hr. following exposure of monkeys to aerosols of *P. pestis* there were no clinical signs of infection. This was correlated with the absence of tissue alterations by either light or electron microscopy during the first 48 hr. of a serial sacrifice experiment. By 72 hr. the animals developed fever and mild tachypnea, and their lungs contained areas of lobular pneumonia. The failure to find organisms in the tissue prior to this time is disappointing but hardly surprising. One reason is that *P. pestis,* like other bacteria administered in an aerosol,[16,17] virtually disappears during the first few hours after exposure. Two hours following the exposure of guinea pigs to an aerosol of *P. pestis,* bacilli

were seen in bronchi and alveoli of the medial portions of the lung near the hilus, but by 5 hr. only a few bacilli could be seen and they were within macrophages. At 24 hr. they were present only in vacuoles of macrophages. They were not traced thereafter.[18] It has been calculated by Smith *et al.*[19] that only 5% of a calculated inhaled dose was recovered by culture from the lungs of mice 16 hr. later, and Meyer [20] reported approximately 10% recovery in guinea pigs 12 hr. after exposure. Applying those figures to this experiment, approximately 100 plague bacilli would have been expected in the lungs at 12–16 hr. When this estimate is considered in light of the problem of sampling for the electron microscope, in which the total volume of tissue included in the 250–500 thin sections examined from each pair of lungs approximated 0.00025 cu. mm., the chance of finding organisms prior to an advanced degree of proliferation is indeed infinitesimal. Thus, the question of the location of the bacilli during the pre-inflammatory stages of infection remains unanswered.

Our primary interest in studying the early stages of pneumonia was to determine the pathogenesis of the edema. Electron microscopy confirmed the earlier finding [1] that a serous exudate precedes the appearance of leukocytes in the tissues and further revealed that the first leukocytes to appear in the interstitial tissue and alveoli possessed intact granules. Thus, it is unlikely that leukocyte permeability factors of the sort described by Movat and co-workers [21] or other lysosomal substances [22] are responsible for the early increase in vascular permeability. Similarly, there is no support for the possibility that mast cells, with their content of heparin and proteolytic enzymes,[23] have a significant influence on the early onset of edema, because the many mast cells found in the adventitia of veins and venules during the early stages had numerous, intact granules. Later in the disease some of the cells displayed empty granules, which is one method for release of their content,[23] but this can only be considered a secondary development.

There was no morphologic evidence for significant vascular damage as an initial response to infection. Although there were a few capillary endothelial cells with swollen, rarefied cytoplasm and others with a variety of intracellular inclusions, similar changes were present in the areas without edema as well and were too uncommon early in the infection to account for the massive outpouring of fluid in some locations. Carbon particles introduced intravenously 3–5 min. before sacrifice and having an average diameter of 250 Å could not be traced through or between endothelial cells of veins or capillaries except where large gaps had opened for the passage of leukocytes or erythrocytes. The failure to

detect defects with carbon is similar to Florey's experience [24] with ferritin in the inflamed colon of the mouse and rat, but differs from Marchesi's observation [25] that colloidal carbon passed through intercellular junctions of venule endothelium in mildly traumatized rat mesentery. An alternative schedule of carbon injections or a tracer having a considerably smaller size, such as peroxidase,[26] would seem more appropriate in future efforts to detect the sites of increased vascular permeability. No increase in the size or number of pinocytotic vesicles was found, as has generally been the case.[27]

The plague bacillus may be directly responsible for edema, without invoking the participation of an endogenous mediator. The precedent for such a possibility was established many years ago for the pneumococcus by Sutliff and Friedemann,[28] who isolated a soluble product of the coccus that provoked edema in both the skin and lungs of dogs and helped to explain the propensity of pneumococcal pneumonia to spread rapidly through an entire lobe. In the case of *P. pestis*, Schär and Meyer [29] used the protein murine toxin from the bacillus to produce in mice by intranasal inoculation a pneumonitis that strongly resembled the early stages of plague pneumonia, with interstitial edema followed by leukocytic exudation, necrosis, and hemorrhage. The murine toxin is relatively nontoxic for monkeys and most other species except mice and rats,[30] but other components of *P. pestis* may have similar effects. Interstitial pneumonitis, characterized by massive alveolar septal edema, foci of capillary hemorrhage, and an infiltrate of neutrophils, mononuclear phagocytes, and eosinophils, is produced in rabbits 24 hr. after exposure to aerosols containing *Escherichia coli* endotoxin.[31] Recent studies have suggested that endotoxin may have a significant role in experimental [13] and human plague [32] by causing intravascular coagulation with disseminated fibrin thrombi. There have been increasing efforts recently to isolate toxic lipopolysaccharides from *P. pestis*.[33] The delay between the onset of a logarithmic growth rate of the organism at 6–12 hr. after infection [34] and the appearance of edema some 48–60 hr. later may be due to the necessity for a certain quantity of toxin to accumulate in the tissues.

The resistance of *P. pestis* to phagocytosis by polymorphonuclear neutrophils has been visualized with the electron microscope. Previous studies have demonstrated that *P. pestis* acquires resistance to phagocytosis by neutrophils after a certain period of infection.[35] Furthermore, neutrophils fail to ingest phagocytosis-sensitive bacilli following exposure to virulent organisms.[35] Plasma from guinea pigs moribund with plague sepsis inhibits phagocytosis of sensitive bacilli by normal neutrophils in vitro.[36] In addition to these acquired factors that impair

cellular responses to infection, the ingestion of bacilli by phagocytes does not insure digestion of the organism. Few examples of morphologic damage to bacilli within macrophages were seen, but many alveolar macrophages containing intact bacilli were found in various stages of degeneration. This substantiates previous conclusions [37] that the resistance of *P. pestis* to intracellular digestion may have greater significance for the virulence of the organism than its resistance to phagocytosis.

The inflammatory and necrotic lesions described in pneumonic plague are of interest because there have been few reports of bacterial pneumonias studied with the electron microscope.[8-10] The changes represent a composite of the various inflammatory processes studied in many experimental situations.[4-7] Many of the pulmonary lesions have been described in viral pneumonitis [38] and toxic edema,[12] and in response to changes in the oxygen content of inspired air.[11] Previous studies of bacterial pneumonia with the electron microscope have dealt with the granulomatous response to tubercle bacilli [8,10] and with the early exudation of leukocytes in mice exposed to pneumococci.[9] The latter report presents photographs of leukocytes passing out of capillaries between endothelial cells, just as in plague, and it mentions, but does not depict, blebs in the alveolar epithelial membrane early in the infection. The possibility that similar electron micrographs are obtained from such a variety of studies because common artifacts result from similar processing techniques is unlikely because there are significant differences as well. For example, osmotic effects may account for alveolar epithelial cell swelling and vacuolation in pneumonic plague, as well as in freshwater drowning,[39] inhalation of carbon monoxide,[40] or poisoning of rats with alpha-naphthylthiourea,[41] but the subendothelial fluid accumulation found in those studies and in staphylococcal enterotoxicity [12] and oxygen toxicity [11] are not found in plague. Therefore, instead of attributing similar lesions to mistreatment of the tissues, it appears more likely that the lung can respond to a variety of noxious stimuli in but a few ways.

Summary

The infection of monkey lungs by *Pasteurella pestis* has been studied with the electron microscope. The disease was first apparent clinically at 60–70 hr. and pathologically at 72 hr. The first tissue response seen with the light microscope was intra-alveolar edema with massive proliferation of organisms. Electron microscopically, the earliest response to the presence of numerous intra-alveolar bacilli was perivenous and peribronchial edema. Interstitial fluid also accumulated in the form of

subepithelial blebs in the thin segments of alveolar septums. Damage to endothelial cells was rare and slight, and intravenous injections of colloidal carbon prior to sacrifice failed to reveal sites of increased vascular permeability. Because leukocytic infiltration followed the accumulation of extravascular fluid and because mast cells and their granules were intact during the early stages of the infection, neither factor appears to have a primary role in the pathogenesis of edema. Once the cellular component of the reaction developed, *P. pestis* was found to resist phagocytosis by polymorphonuclear neutrophils and to resist intracellular digestion by mononuclear phagocytes. Invasion of the tissues by the organism was observed only in the advanced stages of the pneumonia, when all components of the alveolar septums were undergoing necrosis.

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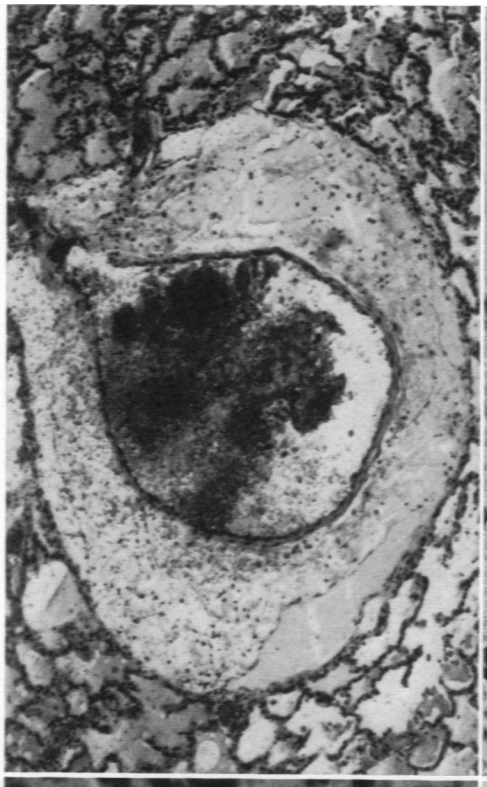
Legends for Figures

Fig. 1. Pulmonary edema. Adventitia of a vein, perivascular lymphatics, and many alveoli are distended by eosinophilic fluid. There is minimal leukocytic infiltration and focal extravasation of erythrocytes. Hematoxylin and eosin. × 68.

Fig. 2. Early lobular pneumonia. Alveoli and alveolar ducts are filled with eosinophilic fluid containing numerous *P. pestis*. Leukocytes are found in modest numbers in septums and alveoli. Septums are intact. Hematoxylin and eosin. × 210.

Fig. 3. Epithelial blebs. Two fluid-filled blebs protrude into alveoli from a septum. The interstitial location of the fluid is demonstrable by electron microscopy (Fig. 6 and 7). Hematoxylin and eosin. × 500.

Fig. 4. Necrotizing pneumonia. Alveolar architecture is obliterated. Neutrophils are abundant and there are colonies of *P. pestis* in perivascular lymphatics. Hematoxylin and eosin. × 210.



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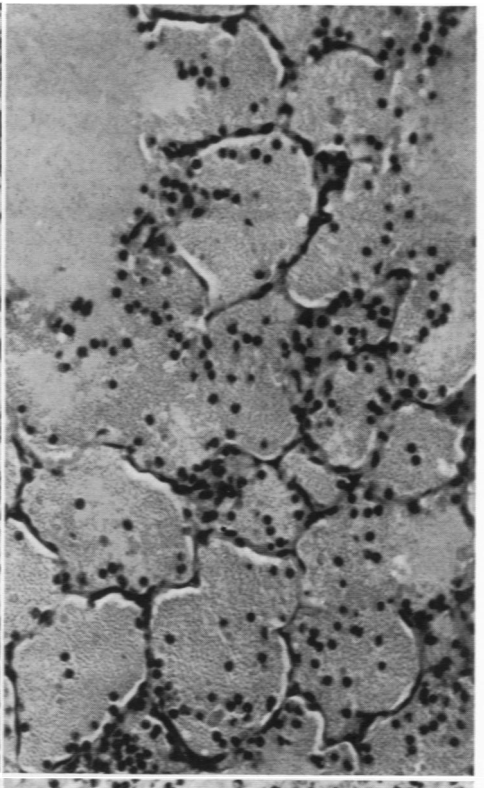

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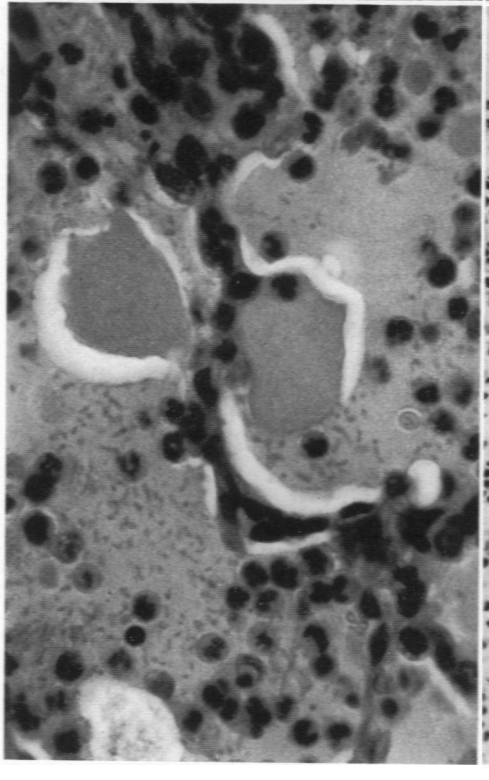

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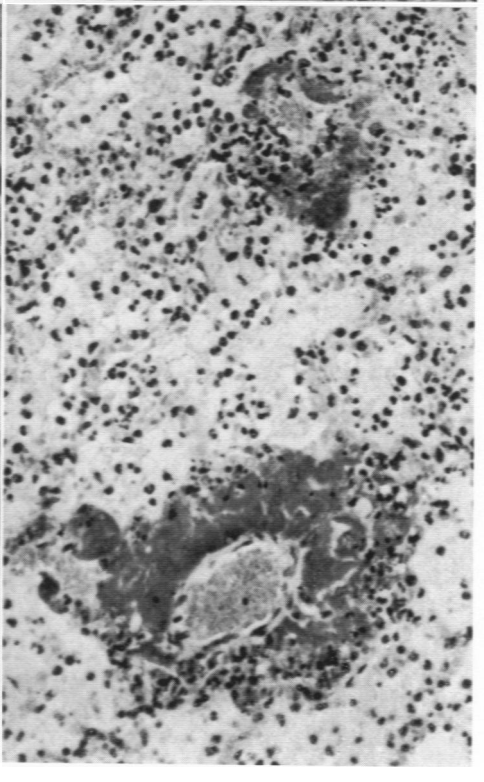

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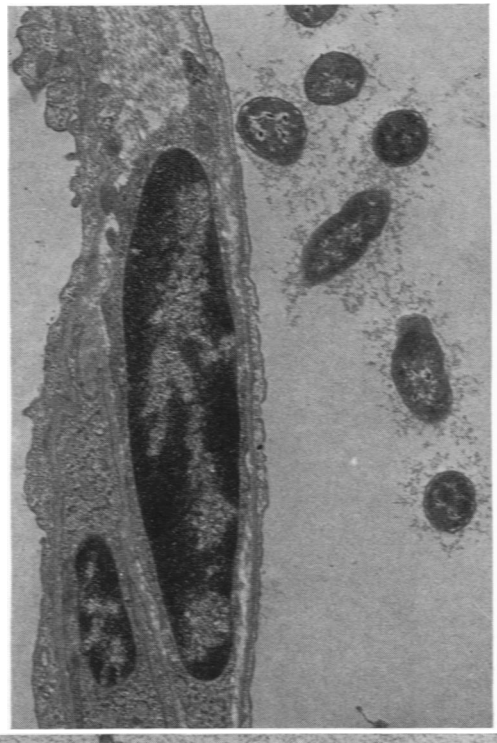
All electron micrographs are of sections treated with a saturated solution of uranyl acetate. *A*, alveolar lumen; *I*, interstitial tissue; *C*, capillary lumen.

Fig. 5. Infected lung. Several *P. pestis* are present in an alveolus near a septum that is entirely normal. × 9000.

Fig. 6. Intra-alveolar bleb. A subepithelial bleb is formed by the dissection of edema fluid between the alveolar epithelial cell and the basement membrane. In this illustration the possibility that the fluid is within an intracellular vacuole cannot be excluded. × 6200.



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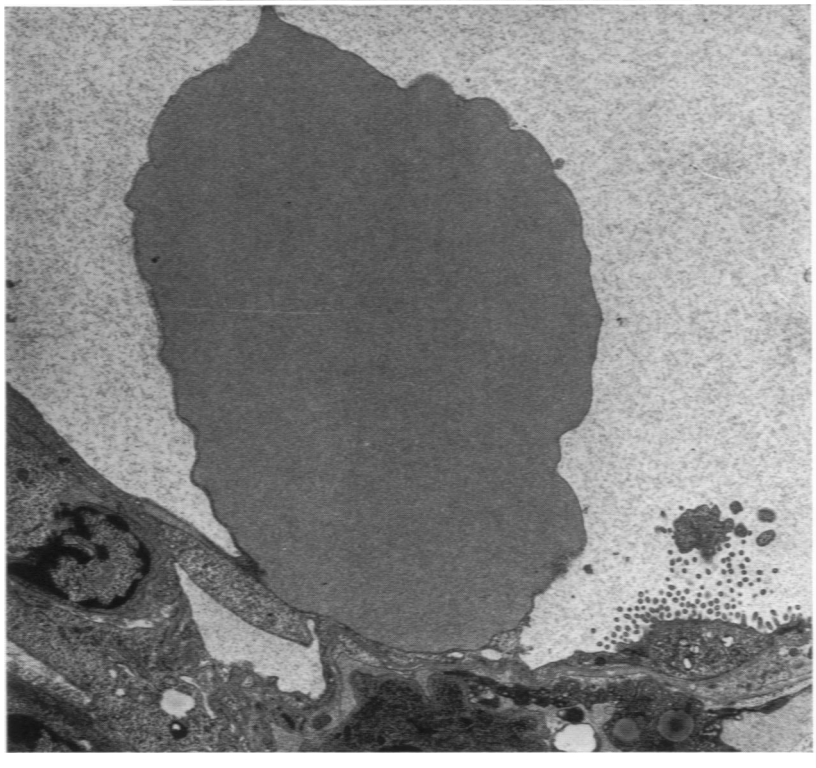

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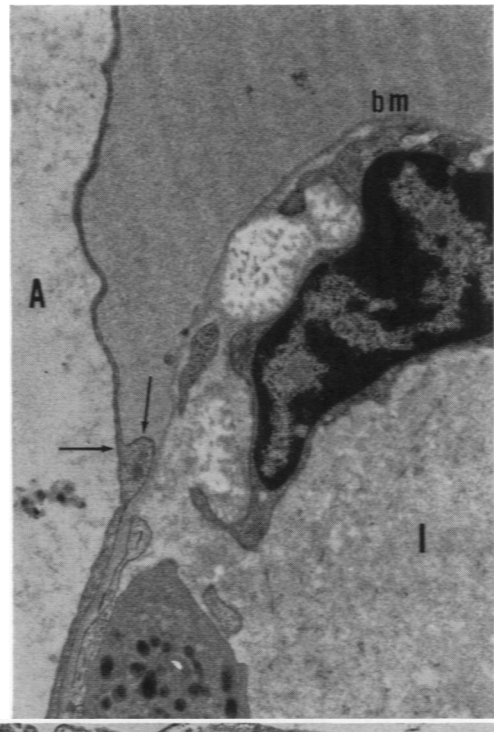
Fig. 7. Intra-alveolar bleb. Higher magnification of a similar bleb clearly reveals the interstitial location of the fluid. Plasma membranes of the epithelial cell are intact *(arrows)* and the epithelial basement membrane *(bm)* is also unaltered. × 8800.

Fig. 8. Necrotizing pneumonia. This section was taken from an area of necrosis seen with the light microscope (Fig. 4). Many cells are fragmented with granular cytoplasm and swollen, rarefied mitochondria. Interstitium is edematous. × 8100.



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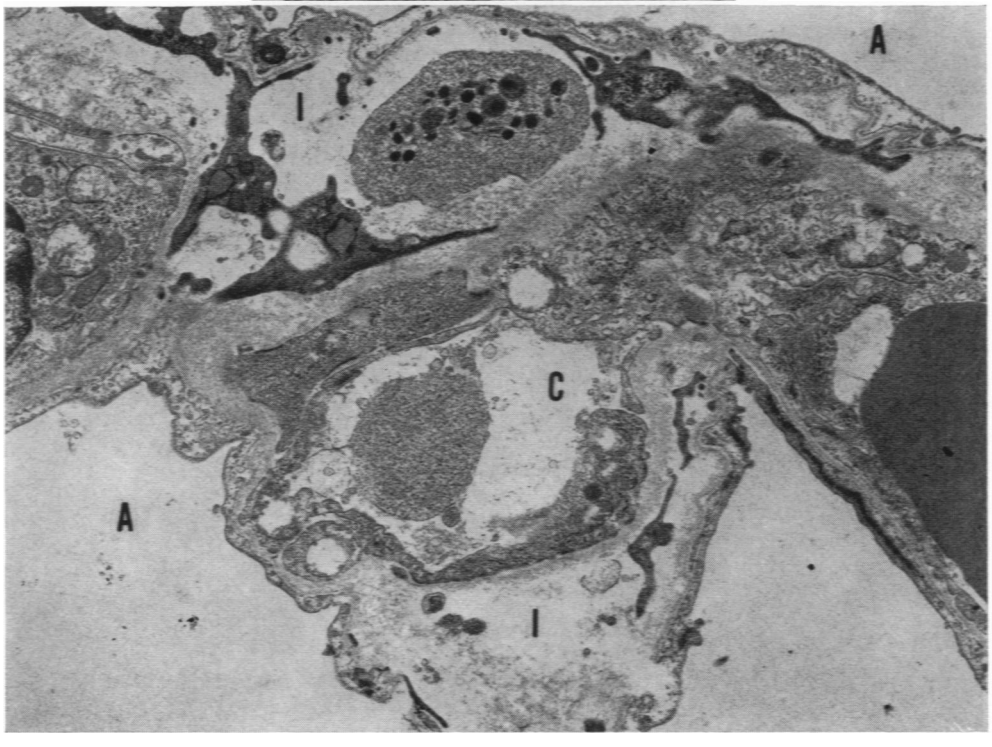

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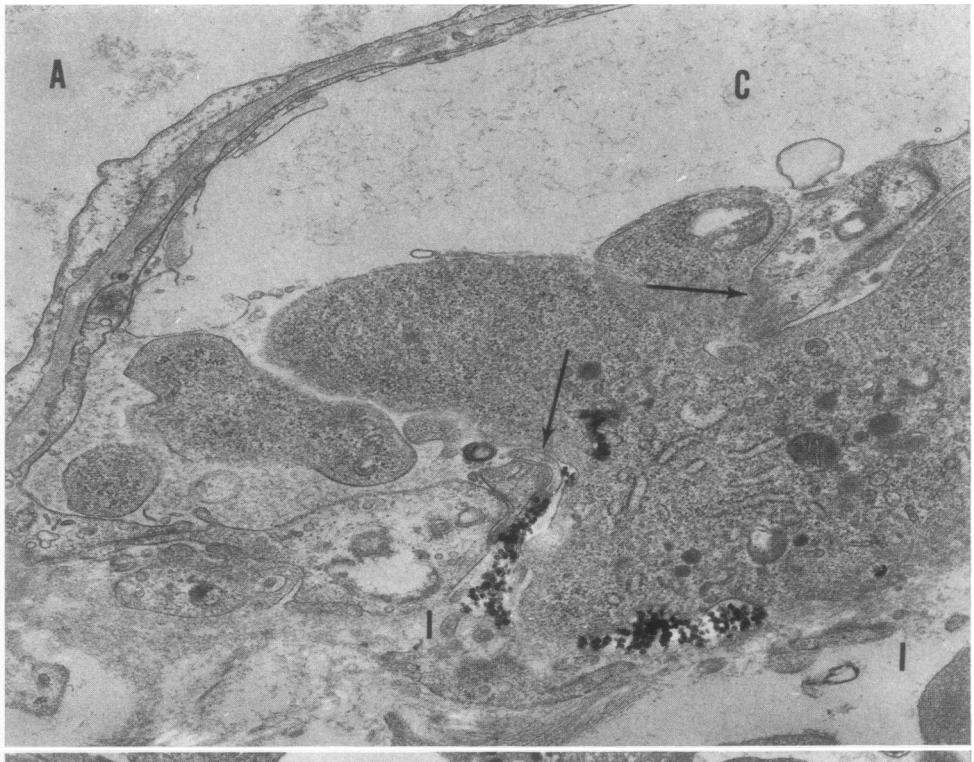

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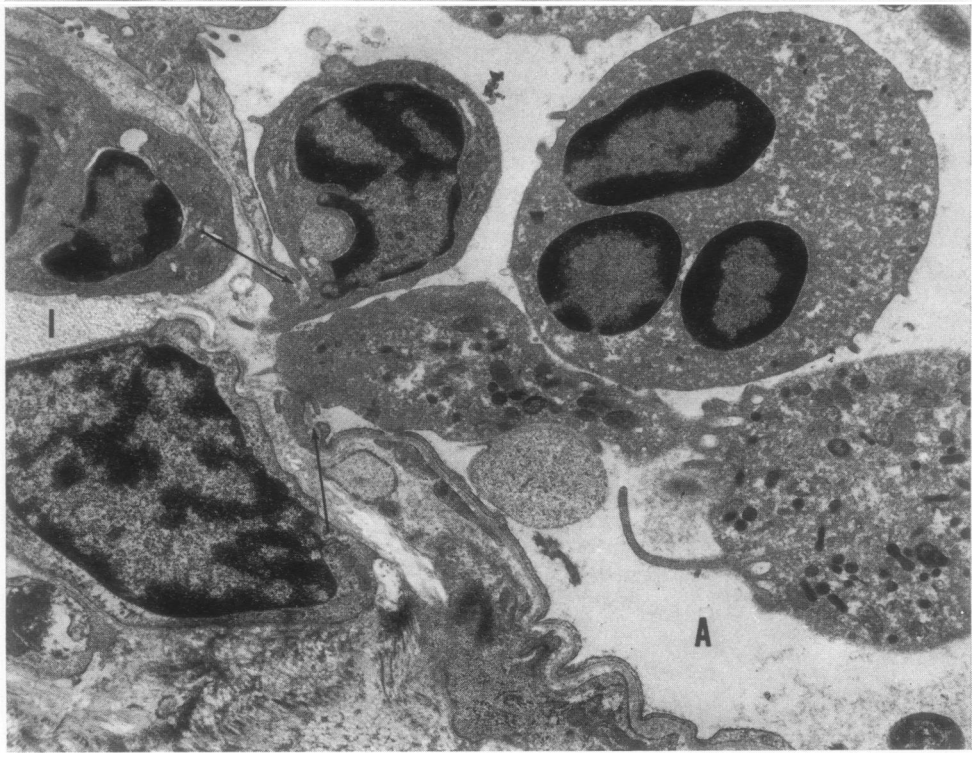

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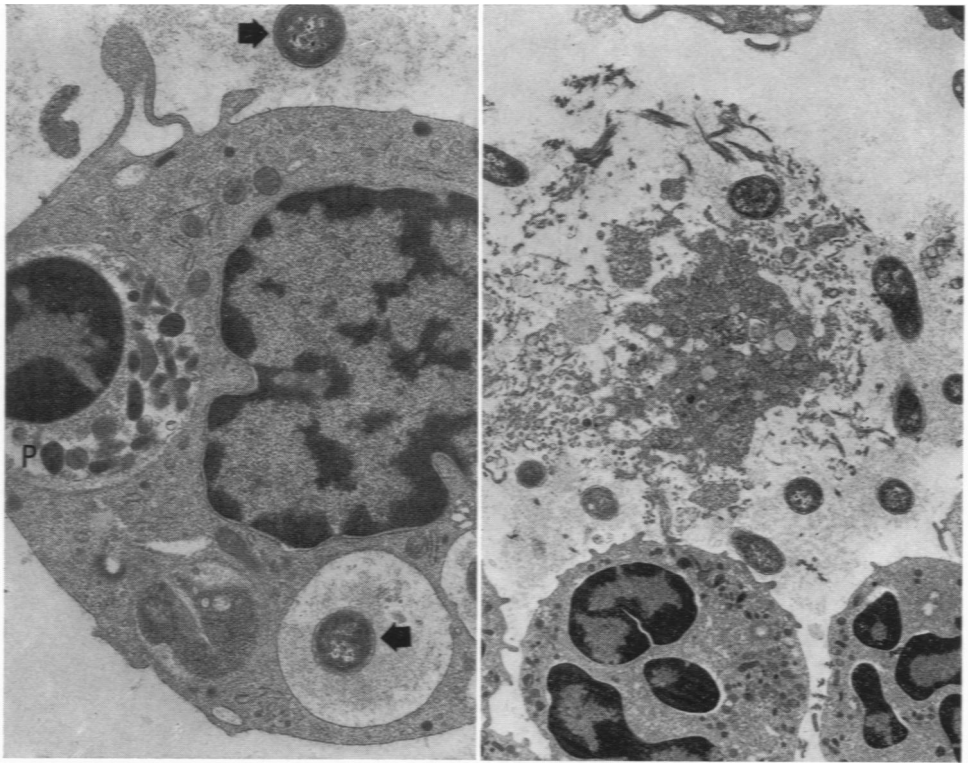

Fig. 9. Leukocyte migration. A gap *(arrows)* in an alveolar septal capillary is occupied by a monocyte, part of which remains in the capillary lumen while part is in the interstitial space. Carbon particles that had been injected intravenously accompany the leukocyte through the endothelial defect. × 16,100.

Fig. 10. Leukocyte migration. There is a gap *(arrows)* in the alveolar epithelial lining, through which two neutrophils are emerging from the interstitium into the alveolar lumen. Portions of epithelial cell cytoplasm in the area appear slightly opaque and granular. Note absence of granules in the polymorphonuclear leukocyte at upper right. This cell is otherwise intact and contains no phagocytized material or vacuoles in this plane of sectioning. × 8000.

Fig. 11. Phagocytosis. An alveolar macrophage has incorporated a neutrophil with intact granules and plague bacilli within phagocytic vacuoles. The intracellular bacillus is identical to one in the alveolar fluid *(arrows)*. Granular material surrounding each bacillus may represent the capsule. Only a few lysosomes (small, round, dense cytoplasmic organelles) are present. × 9700.

Fig. 12. Necrosis of a leukocyte. The debris of a cell in the alveolar exudate is associated with several intact bacilli. × 5000.