

10-1-1966

# Pneumonia due to *Mycoplasma* in Gnotobiotic Mice: II. Localization of *Mycoplasma pulmonis* in the Lungs of Infected Gnotobiotic Mice by Electron Microscopy

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# Pneumonia due to *Mycoplasma* in Gnotobiotic Mice

## II. Localization of *Mycoplasma pulmonis* in the Lungs of Infected Gnotobiotic Mice by Electron Microscopy

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Received for publication 16 July 1966

### ABSTRACT

ORGANICK, AVRUM B. (Marquette University School of Medicine, Milwaukee, Wis.), KENNETH A. SIEGSMUND, AND IRVING I. LUTSKY. Pneumonia due to mycoplasma in gnotobiotic mice. II. Localization of *Mycoplasma pulmonis* in the lungs of infected gnotobiotic mice by electron microscopy. *J. Bacteriol.* 92:1164-1176. 1966.—Lesions in lungs of gnotobiotic mice inoculated intranasally with *Mycoplasma pulmonis* were examined by electron microscopy after osmic acid fixation. At 1 week after infection, mycoplasma cells were found in large numbers in the bronchi at the surface of bronchial epithelial cells and, in smaller numbers, in the alveoli where active phagocytosis by polymorphonuclear leukocytes (PMN) occurred. Cytopathic changes in underlying bronchial epithelial cells, not apparent by light microscopy, were observed. At 3 weeks after infection, mycoplasma cells were rarely seen in the bronchi, and were no longer seen free in the alveolar spaces or within PMN. Lungs examined after glutaraldehyde fixation 1 week after infection confirmed the presence of mycoplasma cells in the alveolar spaces and within phagocytic vacuoles of PMN, but also revealed numerous ring forms within granular pneumocytes. These forms seemed to represent intracytoplasmic developmental stages of *M. pulmonis*, in which elementary bodies appeared in large numbers.

Pneumonia produced by the intranasal inoculation of *Mycoplasma pulmonis* in the gnotobiotic mouse (10) provided the opportunity to search for mycoplasma within the pulmonary lesions of infected mice by use of electron microscopy. This report deals with the localization of mature mycoplasma cells in the bronchi, in the alveolar spaces, and within phagocytic vacuoles of polymorphonuclear leukocytes in an early stage of the infection. What appeared to be developmental forms of *M. pulmonis* were also observed in an intracytoplasmic location in granular pneumocytes.

### MATERIALS AND METHODS

Gnotobiotic Ha/ICR mice (4 to 6 weeks old) of both sexes were inoculated intranasally with  $9.0 \times 10^4$  colony-forming units (CFU) of a strain of *M. pulmonis*. (This strain of *M. pulmonis* was kindly provided by John B. Nelson, Rockefeller University, in Swiss-Webster mice inoculated intranasally with a 30th lung passage, and passed four times in mycoplasma broth.) Control gnotobiotic mice were inoculated with sterile mycoplasma broth. At 1 and 3 weeks after inoculation, mice were removed alive from the isolators within sterile plastic containers,

and sacrificed by traction of the neck upon a sterile field. Lungs were removed aseptically within 2 min of the time respiration ceased, minced in chilled osmic acid fixative, and embedded in Vestopal. Adjacent portions of lung were fixed in glutaraldehyde and embedded in Vestopal W (Martin Jaeger Co., Geneva, Switzerland). Other portions of pneumonic lungs were fixed in Helle's fixative, embedded in paraffin, and stained with hematoxylin and eosin. Thick sections made with a Porter-Blum microtome of the material embedded with Vestopal W were stained with Toluidine blue, and appropriate blocks were selected that contained bronchi and alveoli. Thin sections were prepared for electron microscopy by use of the LKB Ultratome, and stained with uranyl acetate and lead citrate. These were examined with RCA EMU-3g and EMU-3b electron microscopes.

### RESULTS

In ultrathin sections of osmic acid-fixed lungs, mycoplasma cells were seen in large numbers in the bronchi and, in smaller numbers, in the alveoli 1 week after intranasal inoculation. Most striking were the large numbers of mycoplasma cells lying at the surface of bronchial epithelial cells enmeshed among the bases of cilia and micro-

villi. In many places, these formed a continuous blanket or layer, three to five organisms thick, at the surface of some ciliated cells (Fig. 1 and 2). They were less numerous at the surface of non-ciliated or "Clara" cells. Occasionally, small numbers of mycoplasma were seen in the intercellular spaces between adjacent columnar epithelial cells. At no time, however, were mycoplasma cells actually seen within the cytoplasm or within the vacuoles inside of the main portion of bronchial epithelial cells. There was nothing resembling partially digested mycoplasma cells inside of the host epithelial cells. The bronchial epithelial cells showed distention of intracellular spaces and disruption of mitochondria, which represents a cytopathic effect, since this appearance differed from that of control animals (Fig. 1).

Mycoplasma were seen in clusters within the

alveolar spaces, occasionally enmeshed in granular material containing fibrin, in lungs of infected animals 1 week after inoculation (Fig. 3 and 5). Numerous polymorphonuclear leukocytes (PMN) were also present, and they were actively engaged in phagocytosis of mycoplasma cells. Mycoplasma cells were seen not only at the surface of PMN, but also within vacuoles within the cytoplasm of the phagocytes (Fig. 4).

Three weeks after intranasal inoculation, mycoplasma were rarely seen in the bronchi, and were not seen free in the alveoli or within fields of numerous plasma cells in the peribronchial and perivascular spaces (10).

Mycoplasma cells were oval or round, and varied from 0.5 to 1.0  $\mu$  in diameter. They were similar in appearance to electron micrographs of *M. pulmonis* (12) and of other mycoplasmata

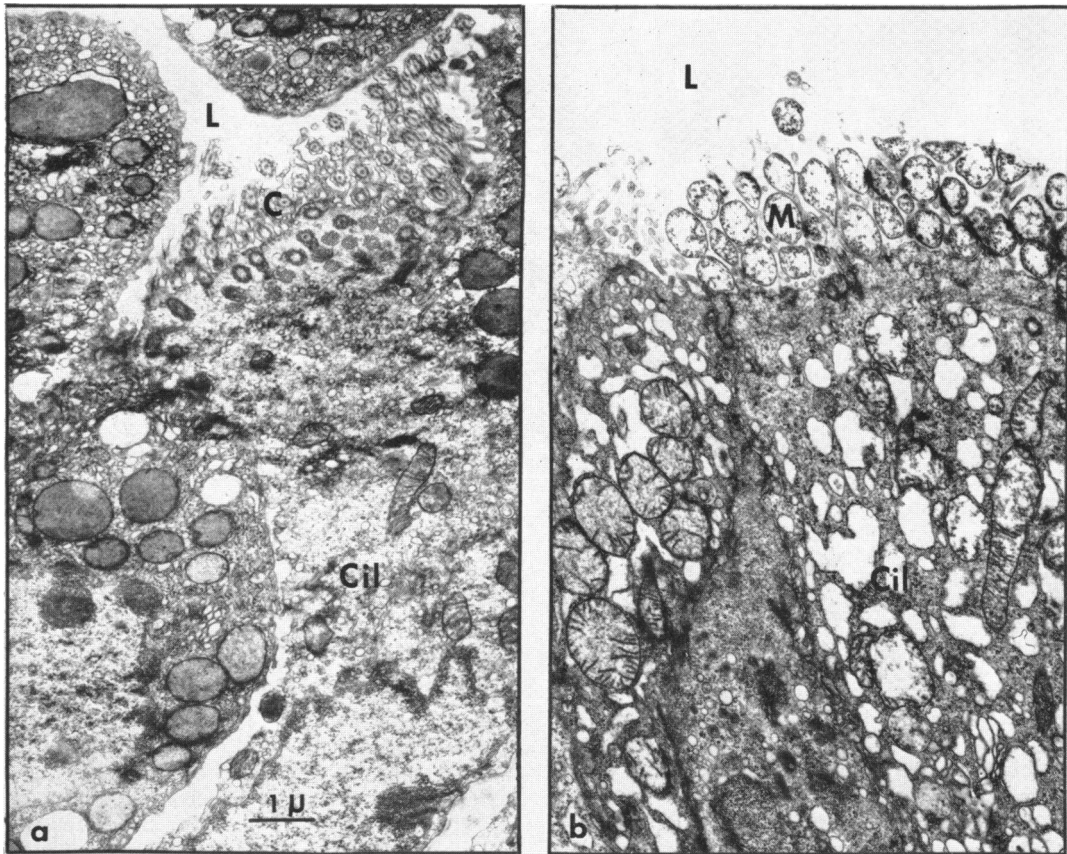


FIG. 1. (a) Bronchus of a normal mouse. Ciliated cells have elongated mitochondria. Cross-sections of cilia rootlets near the surface of the cell and of cilia in the bronchial lumen (L) can be seen. Cells with the large, rounded bodies (mitochondria) are the nonciliated or "Clara" cells. (b) Bronchus of a mouse 1 week after intranasal inoculation with *Mycoplasma pulmonis*; mycoplasma cells (M) are massed two or three deep at the surface of a ciliated cell, but are confined to the lumen. Clear spaces within the underlying bronchial cell represent cytopathic effects: distended intracellular spaces and partially disrupted mitochondria. Osmic acid fixation;  $\times 15,000$ .

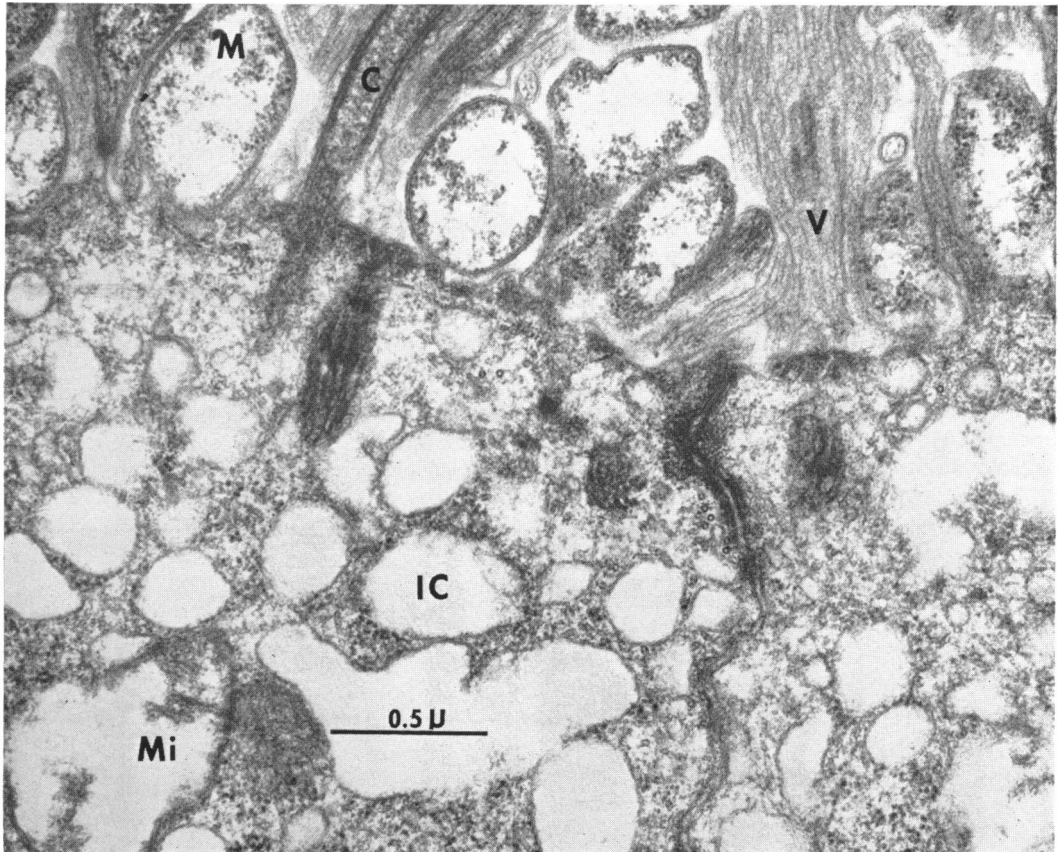


FIG. 2. *Mycoplasma* cells (*M*) at the surface of two adjacent ciliated bronchial epithelial cells 1 week after intranasal inoculation. *Mycoplasma* cells are closely apposed to the surface of the host cell. They are wedged between the bases of cilia (*C*) and microvilli (*V*), but do not penetrate the body of the epithelial cell. Disruption of mitochondria (*Mi*) and distention of intracellular spaces (*IC*) are seen. Osmic acid fixation;  $\times 72,000$ .

(3, 5) with the following features: (i) a triple-layered unit membrane; (ii) nucleoids in which clear areas were traversed by electron-dense strands; and (iii) ribosomes (Fig. 2-5). In the alveoli, in addition to the more common type of cells, some of the mycoplasma had a more finely granular, dense cytoplasm without clear areas (Fig. 3), and were similar to those described in *M. hominis* (1). In addition, occasional bizarre, irregular, convoluted forms were also seen (Fig. 5), which may represent unusual extracellular forms of mycoplasma.

At 1 week after intranasal inoculation, portions of pneumonic lungs which had been fixed in glutaraldehyde revealed a variety of intracytoplasmic forms (Fig. 6-10). These consisted principally of dense, open or closed ring forms (which probably represent sections of deep pouches or sacs). The rings themselves consisted of a compound membrane approximately 400 Å

thick (Fig. 6-8 and 10). A very dense innermost layer, 120 Å thick, was flanked on either side by alternating light, dense, light, and dense layers, 50, 45, 30, and 20 Å thick, respectively (Fig. 11). In close association with the ring forms, most often appearing within the concavity of the ring (or pouch), were found large numbers of small, extremely dense, membrane-limited, rounded bodies, the smallest of which (50 to 80 μ) resembled elementary bodies (Fig. 6-10). The probable manner of formation of the elementary bodies was observed in numerous examples. A portion of the ring, usually near the open end, would appear widened due to the interposition of dense material between the leaves of the compound membrane. Numerous elementary bodies were formed by segmentation of the dense material and separation of numbers of elementary bodies (Fig. 10a). Elementary bodies within the concavity of the ring (or pouch) exhibited a wide

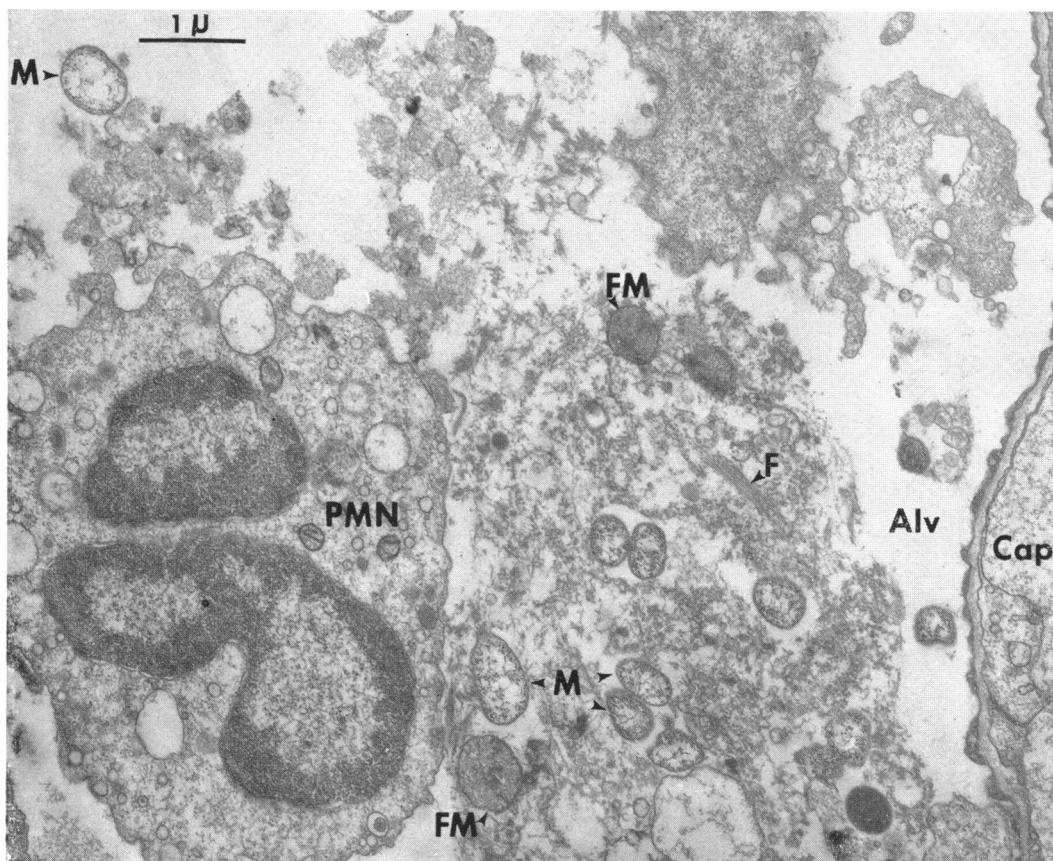


FIG. 3. Exudate in the alveolus (Alv) of the lung of a mouse with pneumonia 1 week after intranasal inoculation with *Mycoplasma pulmonis*. A portion of a capillary (Cap) is seen on the right. A polymorphonuclear leukocyte (PMN) and masses of granular material containing fibrin strands (F) occupy the alveolar space. Numerous mycoplasma (M) are trapped in the fibrin-containing mass, and some are free in the alveolar space. Finely granular mycoplasma cells (FM) are present in moderate numbers. Osmic acid fixation;  $\times 24,000$ .

variety of sizes. The larger ones usually had a less dense, but still fairly uniform, granular cytoplasm. Within the same host cell as the ring forms, there were often seen numbers of larger round or ovoid bodies approximately  $0.5 \mu$  in diameter (Fig. 6 and 7). These had distinct unit membranes and uniform, finely granular cytoplasm without nucleoids, which appeared to be similar to, or the same as, the finely granular form of mycoplasma (M) found in the alveolar spaces. Thus, it appeared that various stages of a mode of reproduction of what was probably *M. pulmonis*, and which involved elementary bodies, were all represented in the same host cell.

The ring structures in these ultrathin sections were often exceedingly complex. At times, an outer ring enclosed a second inner ring. At other times, rounded or clubbed tips of shorter membrane-enclosed structures projected into the

concavity of the ring structure, suggesting portions of microtubules (Fig. 7, 8, and 10b). The feature common to all of these intracytoplasmic forms, the ringlike compound membrane, can best be interpreted as a section through a pouch. It is unlikely that random sections of slender microtubules would give so many nearly perfect rings or segments of rings. The incompleteness of the rings suggested that the sacs or pouches had open mouths. High magnification views of these structures further suggested that they were open-mouthed, because elements of the host-cell cytoplasm, including ribosome-like bodies, were seen with approximately equal frequency within the concavity of the ring as they were outside of the ring (Fig. 7 and 10b).

The host cells involved in this process were the large alveolar cells containing dense, lamellated bodies, the "dust" cells (13), or the "type 2

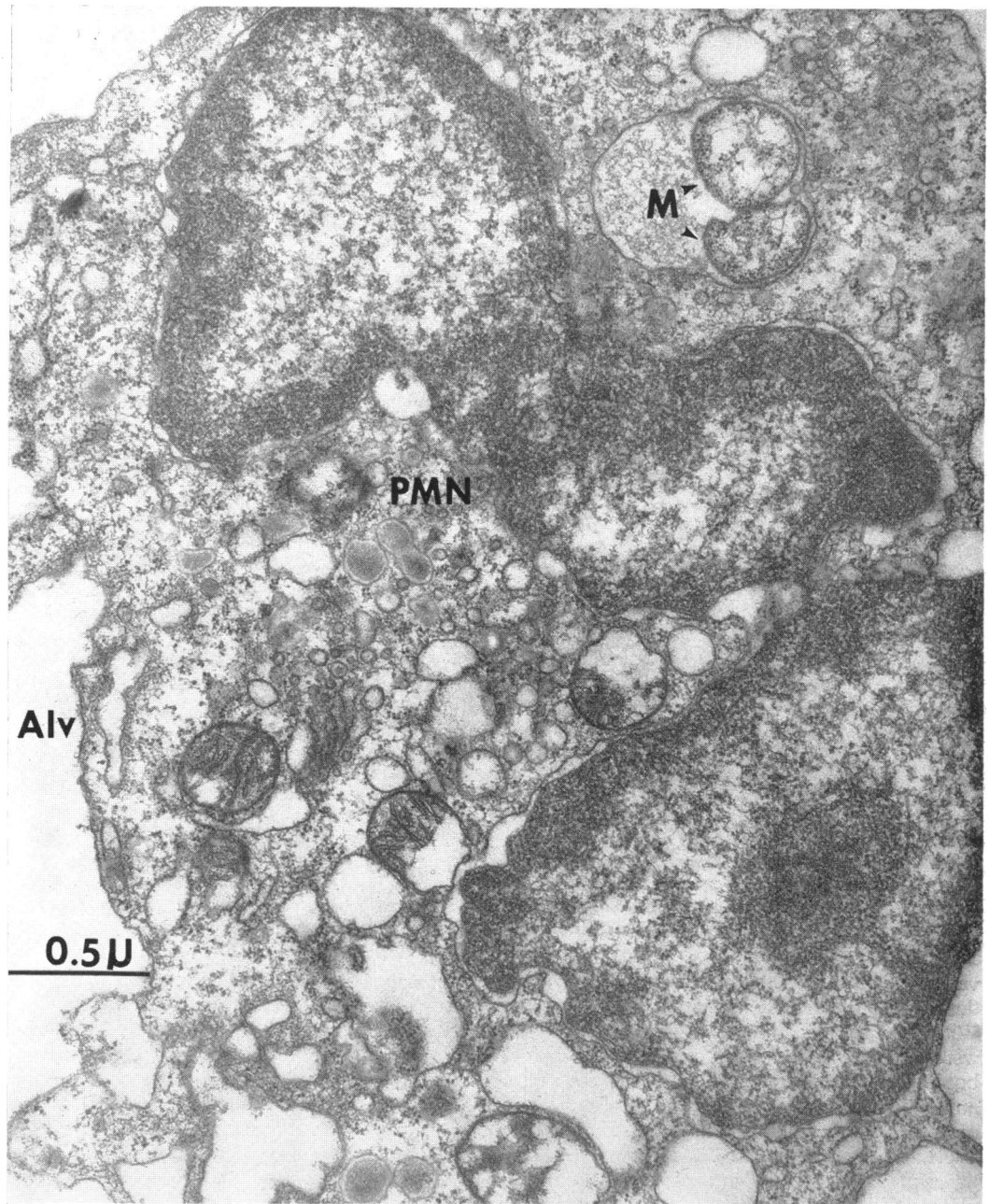


FIG. 4. Two mycoplasma cells (*M*) are seen within a single phagocytic vacuole in a polymorphonuclear leukocyte (*PMN*) in the alveolus (*Alv*) of the pneumonic lung of a mouse 1 week after intranasal inoculation with *Mycoplasma pulmonis*. Osmic acid fixation;  $\times 56,000$ .

alveolar pneumocytes" or "granular pneumocytes" (8). The presence of mature mycoplasma cells in the lumen of the alveoli and within the PMN vacuoles was confirmed in the glutaralde-

hyde-fixed material. The finding of mycoplasma in the PMN and of ring forms in granular pneumocytes represented two different processes. In PMN, previously formed mycoplasma cells were

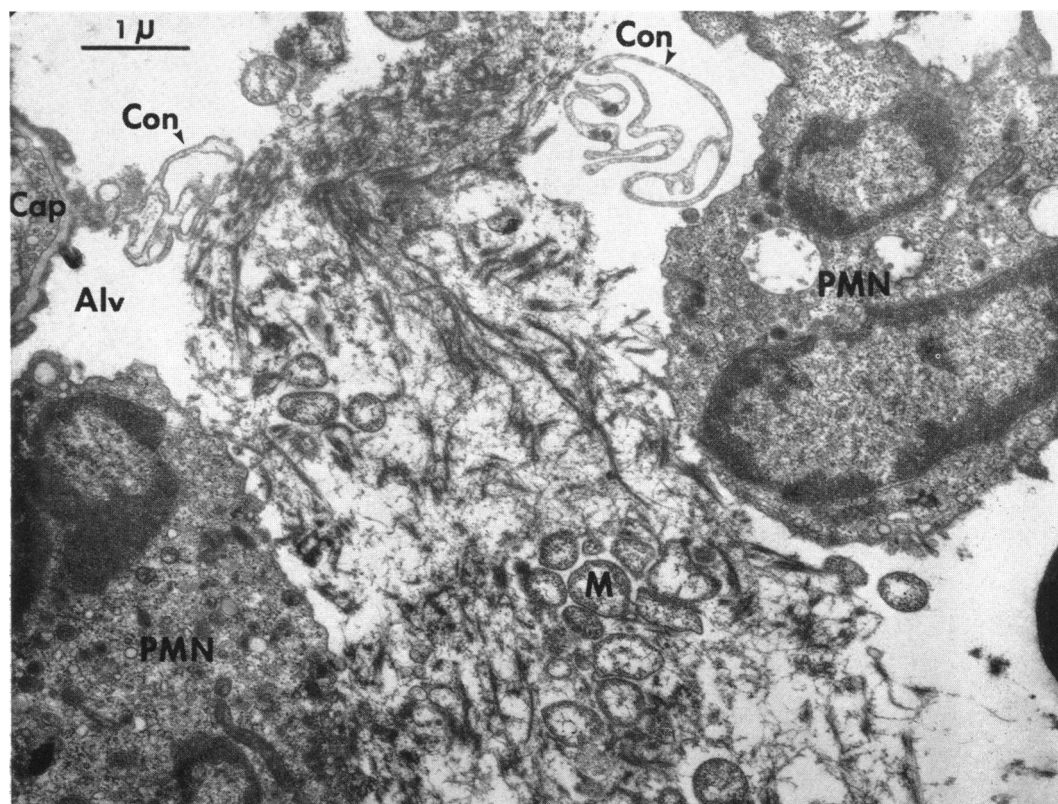


FIG. 5. Bizarre, convoluted, membranous structures (Con) in the alveolar exudate in the lung of a mouse with pneumonia 1 week after intranasal inoculation with *Mycoplasma pulmonis*. Numerous ovoid mycoplasma cells (M) are visible within the fibrin-containing mass in the same region. Osmic acid fixation;  $\times 24,000$ .

in the process of being phagocytosed. In the case of the ring forms within the granular pneumocytes, the process seemed to be the formation of new mycoplasma cells. Review of the osmic acid-fixed material revealed similar ring forms within granular pneumocytes in lungs of mice 1 and 3 weeks after intranasal inoculation with *M. pulmonis*.

#### DISCUSSION

In experimental studies with *M. pneumoniae* in the chick embryo (9) and in hamsters (2, 7), antigen was found localized at the surface of the bronchial epithelium. This localization was demonstrated by immunofluorescence techniques and by aniline dye techniques, Giemsa stain (7) and the modified Brown and Brenn stain (2). In all of these instances, the underlying bronchial epithelium appeared normal in stained sections by light microscopy, and some of the bronchial epithelial cells appeared to contain mycoplasma within their substance as well as at their surface.

None of the investigators was able to find microbes in the alveoli or anywhere except the bronchial epithelium.

This report represents the first demonstration by use of electron microscopy of mycoplasma in an experimentally infected intact animal. These studies confirm the fact that mycoplasma are present at the surface of bronchial epithelial cells in experiments with a respiratory-tract pathogen in an appropriate species. They provide at higher magnifications two points of clarification of the relationship between mycoplasma and the underlying bronchial epithelial cell. In the model used in this experiment, *M. pulmonis* in the gnotobiotic mouse, mycoplasma cells were distinctly confined to an extracellular position with respect to the bronchial epithelial cell. Their presence in such compact masses at the bronchial epithelial surface explained the relative ease with which they were detected by previous investigators using light microscopy with both immunofluorescence and aniline dyes. The elec-

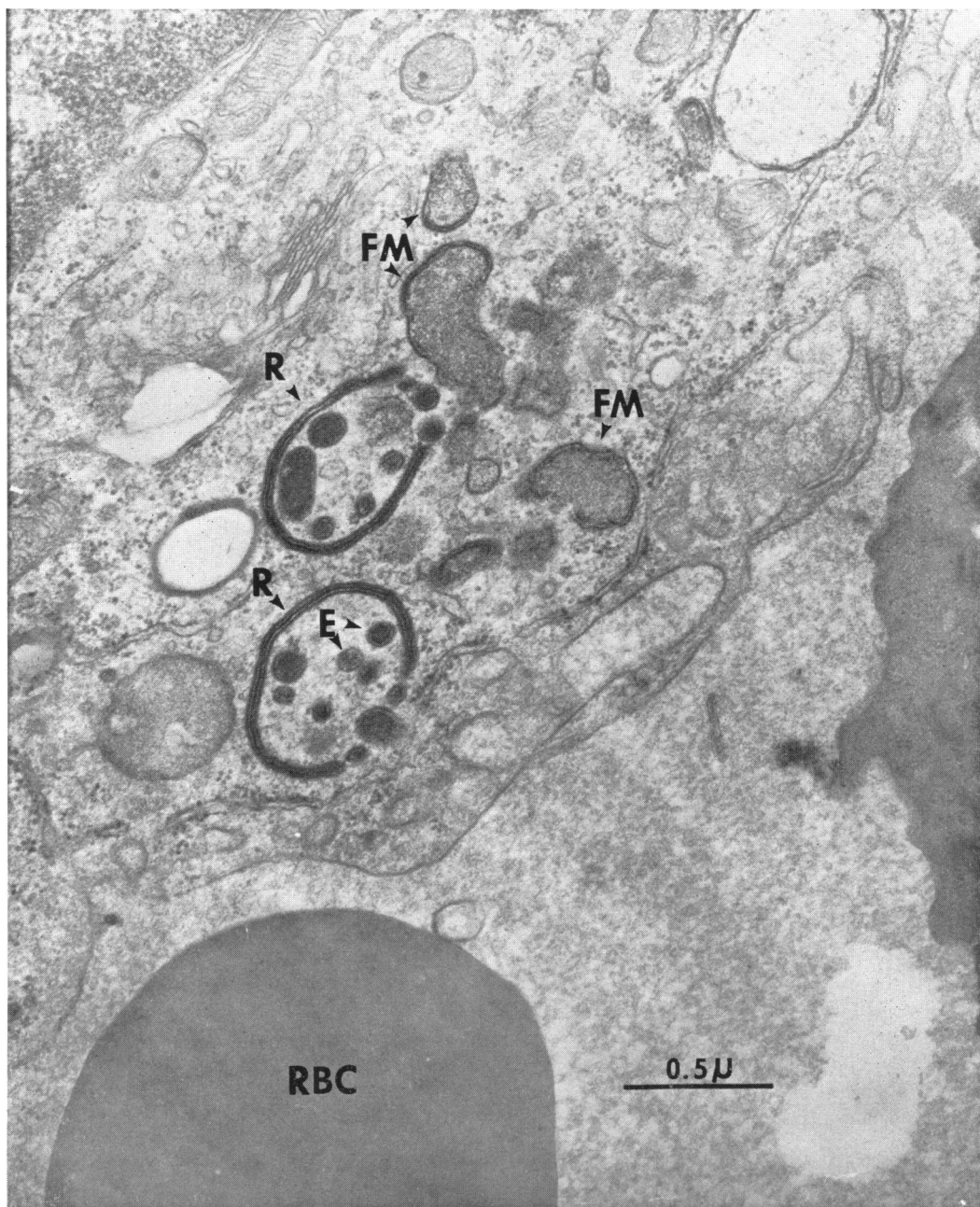


FIG. 6. Intracytoplasmic ring forms (R) from the lung of a gnotobiotic mouse 1 week after intranasal inoculation of *Mycoplasma pulmonis*. The nature of the ring as a compound membrane and the numerous, dense, round elementary bodies (E) both at the margin of the ring and within the concavity of the ring can be seen. Within the same host cell, finely granular, membrane-limited bodies (FM) are also seen. Glutaraldehyde fixation;  $\times 60,000$ .

tron micrographs also demonstrate the extremely intimate contact that the mycoplasma cells have with the surface of the host bronchial cell and with its appendages. It is easy to see, there-

fore, that some degree of overlap of the mycoplasma cell mass could be interpreted as actual invasion of the underlying cell. But the electron micrographs quite clearly settle the point: myco-



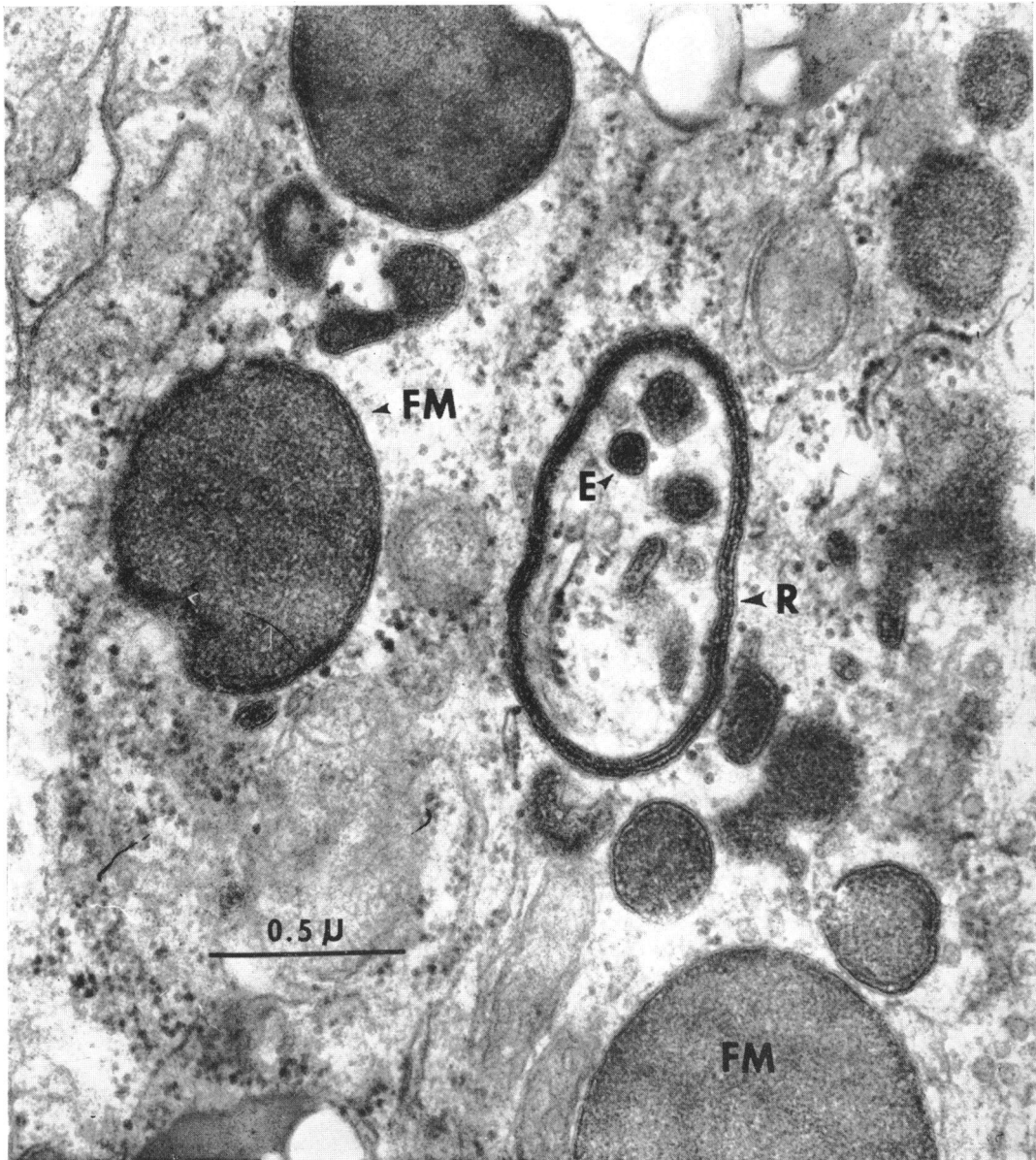


FIG. 7. Intracytoplasmic ring form (R) and finely granular, membrane-limited bodies (FM) from the lung of a gnotobiotic mouse inoculated with *Mycoplasma pulmonis*. In this example, the ring appears complete and contains several elementary bodies (E) of different size. Gradations in size between elementary bodies within the ring and larger, finely granular, membrane-limited bodies (FM) are seen. Glutaraldehyde fixation;  $\times 74,000$ .

plasma do not invade the body of the bronchial epithelial cells.

The second point of clarification the electron micrographs provide is that cytopathic effects (distention of intracellular spaces and disruption of mitochondria) were quite regularly seen, whereas they were not discernible in light-microscopy sections. The functional significance

of these cytopathic changes is probably very great. The large collections of mycoplasma at the surface of the ciliated cells suggest that ciliary action is impaired. The conspicuousness of PMN exudate, which often occludes the bronchus in all stages of the infection in light microscopy (4, 10, 11), may represent further evidence of the failure of the clearing function of the cilia.

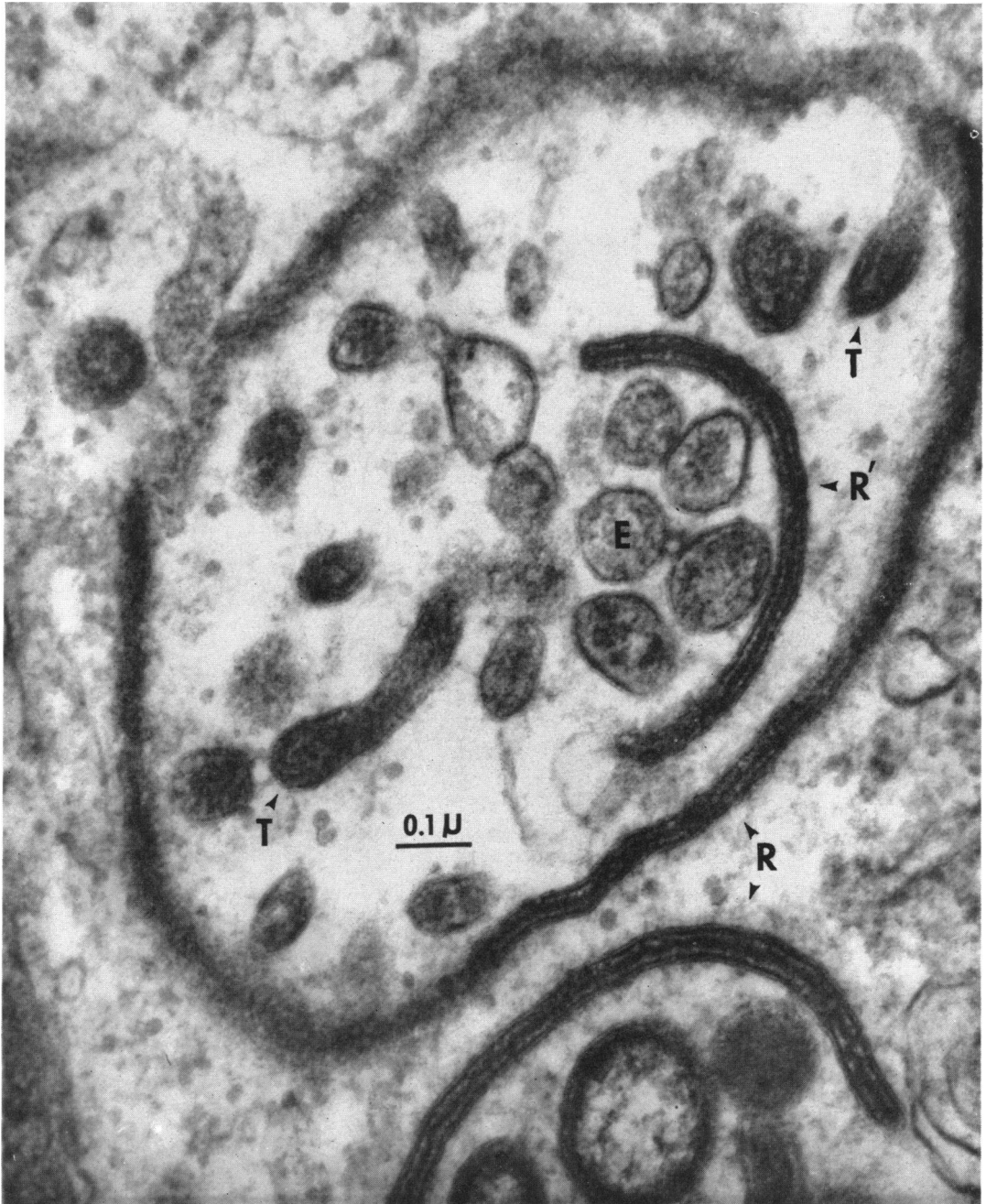


FIG. 8. Complex ring form (R) enclosing a short segment of an inner compound membrane ring (R'). Elementary bodies (E) and portions of what may be tips of microtubules (T) are also seen. Glutaraldehyde fixation;  $\times 150,000$ .

With regard to events in the peripheral portions of the lung, electron micrographs demonstrate what has not been shown in the past: namely, that mycoplasma are present deep in the alveoli as well as at the bronchial epithelial

surface. Within the alveoli, the PMN are actively engaged in phagocytosis. The presence of PMN in the alveoli in the acute stage of the infection is thereby explained. It would appear that, in the acute stage of the experimental pulmonary in-



FIG. 9. Granular pneumocyte (GPN) in the alveolar space (Alv) of a gnotobiotic mouse 1 week after intranasal inoculation with *Mycoplasma pulmonis*. The large, dense, lamellated body (Lam) identifies the cell. A ring form (R) with elementary bodies occupies another portion of the cytoplasm of the same cell. Glutaraldehyde fixation;  $\times 20,000$ .

fection with mycoplasma, polymorphonuclear leukocytes perform a phagocytic function similar to that performed by the phagocytes in acute bacterial pneumonias. Although mycoplasma

were seen in moderate-sized clumps in the alveoli in some of the sections, they were most often seen singly or in very small groups. Nowhere were they seen in so compact and nearly con-

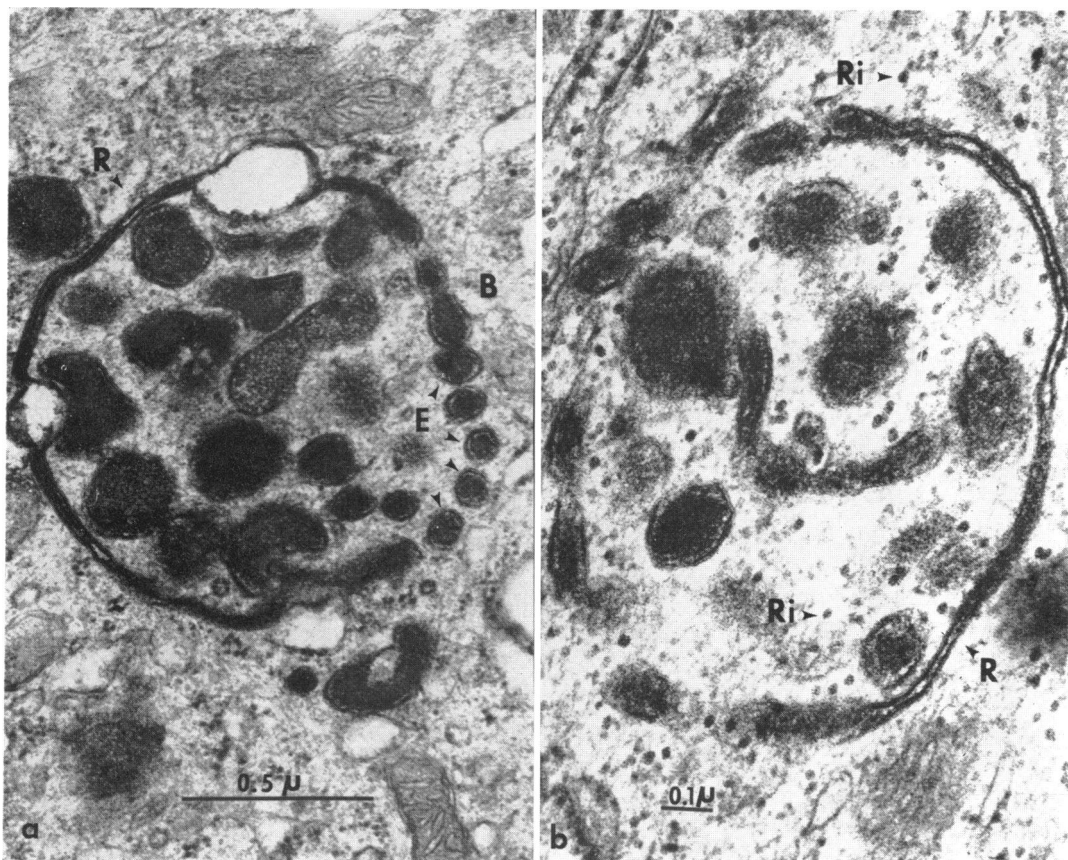


FIG. 10. (a) Ring form (R) containing many dense bodies of intermediate size. The probable manner of formation of elementary bodies is illustrated in this section; a bulbous portion of the ring, widened by expansion of the innermost dense layer of the compound membrane, shows evidence of segmentation (B). Fully formed elementary bodies (E) result from completion of the segmentation process. Each elementary body is enveloped by a unit membrane. A clear zone separates the unit membrane from the central dense portion of the elementary body. Glutaraldehyde fixation;  $\times 90,000$ . (b) Ribosomal elements (Ri) of the host-cell cytoplasm are found within the concavity of the ring form (R) approximately as frequently as they are found outside of the ring. Glutaraldehyde fixation;  $\times 120,000$ .

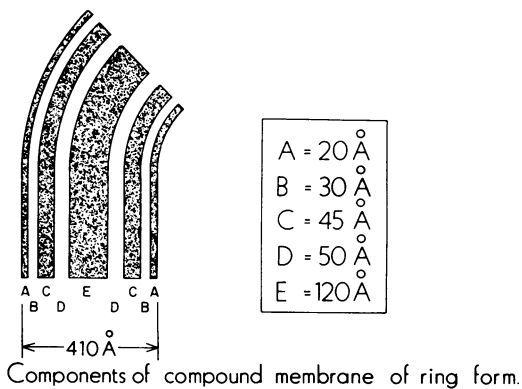


FIG. 11. Components of compound membrane of ring form.

tiguous masses as they were in the bronchial lumen. This difference in distribution may be part of the explanation for the failure of immunofluorescence methods to detect *M. pneumoniae* in peripheral portions of the lung. Experimental infections with *M. pulmonis* (4, 10, 11) differ from experimental infections with *M. pneumoniae* (2, 7), in that pneumonia, often overwhelming, was so frequently produced. It is not surprising, therefore, that the number of infectious organisms is greater in peripheral portions of the lung in *M. pulmonis* infections. Nelson (11) and Edward (4) were able to demonstrate coccobacillary bodies (intracellularly in PMN and free) in impression smears from the cut surfaces of pneumonic lungs in their experiments with murine mycoplasma. It was not pos-

sible to ascertain from that method, however, whether the coccobacillary bodies and PMN were originally present in the bronchi or in peripheral portions of the pneumonic lung.

A recent study (12) of the morphology of *M. pulmonis* by use of electron microscopy with negatively stained whole mounts as well as glutaraldehyde-fixed material in ultrathin sections demonstrated striking pleomorphism of this species. This report, as well as the recent studies of pleomorphism in *M. hominis* species (1), suggests that different modes of reproduction of any species exist, and that the manner of reproduction is probably dependent upon environmental conditions. In our experiments, in which mycoplasma were observed in the lung of a susceptible host and where they were the cause of significant and often fatal disease (10), striking pleomorphism was also observed. Round to oval mycoplasma cells, approximately 0.5 to 1.0  $\mu$  in diameter and possessing an internal structure and limiting membrane similar to those described in ultrathin sections of osmic acid-fixed mycoplasma in cell-free media and in certain tissue cultures described by Edwards and Fogh (5) and by Domeruth et al. (3), were seen in extracellular locations in large numbers and within phagocytic vacuoles. All of the mycoplasma in the bronchus were of this type. Multiplication in the bronchus (if multiplication does take place here) presumably would have taken place in such a manner that daughter cells resemble their parent cells. In the alveoli, considerable variation in morphology of mycoplasma forms exists. A second type of mycoplasma cell, that with a more uniformly granular cytoplasm and absence of clear zones, was seen in small numbers. In addition, a number of bizarre, irregular, and convoluted forms were seen free in the alveoli. In the cytoplasm of the granular pneumocytes, site of the ring forms, the evidence suggests a mode of reproduction which involves the production of elementary bodies and progression through intermediate stages to the larger mycoplasma cells with granular cytoplasm similar to those seen in the alveoli. Although in the material available mycoplasma cells of this type were not seen near the surface of, or emerging from, the host alveolar cell, it is tempting to propose that such a mechanism does, in fact, exist, and that the finely granular mycoplasma cell found in the alveoli is the product of this different mode of reproduction.

Finally, one must consider that in the natural host, as is the case in these studies, only one mode of reproduction exists; that reproduction occurs exclusively in the intracellular location through the complicated mechanism of elementary body formation; that an intermediate stage (the finely

granular stage) is extruded into the alveoli; and that surviving mature mycoplasma cells reach the bronchi in large numbers with no actual multiplication taking place in this location.

The densely staining intracytoplasmic ring forms observed to best advantage in the glutaraldehyde-fixed ultrathin section have never, to the authors' knowledge, been described. Although pouches have been pictured in the extremely pleomorphic *M. pulmonis* in cultures, they did not appear to have the double wall; nor did the mouths of the pouches appear to be the primary site of elementary body formation, as appears to be the case in the unusual forms found in the intracytoplasmic location in vivo. Compound membranes have been pictured as portions of the walls of vacuoles and as portions of tail-like extensions of some *M. hominis* cells (1). It is possible to speculate that the unit membrane of a globular structure could invaginate and fold upon itself to become a double-walled pouch. A layer of dense material (flanked on either side by a less dense layer) enclosed between the two walls of the pouch could produce the compound membrane seen in the ring forms in our studies (Fig. 11). The outermost three layers of the compound membrane are the same size as the outermost three layers of elementary bodies (Fig. 10a) and of mature mycoplasma cells. In *in vitro* studies, elementary bodies seem to form by segmentation of condensed material between the membranes of microtubules (12) or at the tips of mycelial elements (6); thus, it does not seem difficult to imagine a somewhat similar process occurring at the margin or lip of a double-walled pouch. Figure 10a illustrates this process to best advantage.

It is obvious that a true understanding of the nature of so complex a structure as these intracytoplasmic forms can only be achieved by a study of many specimens taken at frequent intervals after inoculation of *M. pulmonis*. The gnotobiotic mice used in this study provide at least some degree of assurance that the test animals are free from other microbes whose presence might seriously interfere with interpreting the results.

#### ACKNOWLEDGMENTS

This investigation was supported by a grant from The American Thoracic Society, Public Health Service general research support grant 5 SO1FR 5434, Training Grant 5T1HE 5538-05 from the National Institutes of Health, and Public Health Service grant AM 4150-05 from the National Institute of Arthritis and Metabolic Diseases.

The encouragement of Paul Kimmelstiel, Ross C. Kory, William W. Stead, and Shirley A. Johnson is gratefully acknowledged. Clelia Johnson, Harlan J. Pederson, Linda M. Fredell, Patricia J. Delaney, and

Constance Wittig provided invaluable technical assistance.

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