Mycoplasma pneumoniae in Hamster Tracheal Organ Culture: Immunofluorescent and Electron Microscopic Studies[1] (35313)

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Hamster trachea in organ culture has been used to study the pathophysiological effects of *Mycoplasma pneumoniae* on the "target cell" of this pathogen, the ciliated respiratory epithelium (1). In this model system, *M. pneumoniae* produced disturbance of ciliary motion and cytopathologic changes that were not seen with other human mycoplasmas tested (*M. hominis, M. salivarium, M. pharyngis, M. fermentans*). To provide further information on pathogenetic mechanisms, these studies have been extended to determine the degree and nature of host cell parasitism by *M. pneumoniae*. This study concerned examination of infected tracheal organ cultures by immunofluorescence and electron microscopy.

Materials and Methods. Hamster tracheal organ cultures were prepared and maintained in Hayflick's medium (2), and observed for ciliary motion as previously described (1). The organ cultures were inoculated with virulent *M. pneumoniae* strain M129 isolated from a patient with pneumonia. The initial organism concentration in the organ culture fluid was $10^{6.15}$ colony-forming units/ml. The control organ cultures received sterile broth as inocula.

For immunofluorescence tracheal rings were removed from the organ culture dish, quick-frozen in O.C.T. compound (Ames Co., Elkhart, Indiana), and 4-μ sections were cut in a microtome cryostat. The sections were treated with rabbit *M. pneumoniae* antiserum followed by goat antirabbit globulin conjugated with fluorescein isothiocyanate as described previously (3). For electron microscopy, comparable tracheal rings were removed from the organ cultures and fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.3) for 30 min at 4°. The tissue was then washed and secondary fixation was carried out in 1% OsO_4–Veronal acetate buffer (pH 7.3) for 90 min at 4°, followed by dehydration in acetone and embedding in Vestopal W (Mme. Martin Jaeger, Geneva, Switzerland). Thin sections were cut on a Reichert ultramicrotome using glass knives and stained with uranyl acetate (4) and lead hydroxide (5). The sections were examined in an Akahsi TRS-50EI electron microscope.

Results. In previous studies using light microscopy, the localization of *M. pneumoniae* in respect to damaged hamster tracheal cells could not be resolved. However, late in the course of infection, small granular structures resembling mycoplasma colonies were noted on the epithelial border (1). To define earlier changes and provide specific identification of these structures, studies were made of infected organ cultures by immunofluorescent methods and electron microscopy.

Immunofluorescent studies. A series of hamster tracheal sections was prepared, established in organ culture, and inoculated with *M. pneumoniae*. At daily intervals, corresponding to stages of epithelial damage previously described (1), a specimen was observed for ciliary motion, then removed and processed for immunofluorescent study. At 24 hr, when ciliary motion appeared unchanged, little specific fluorescence could be identified.

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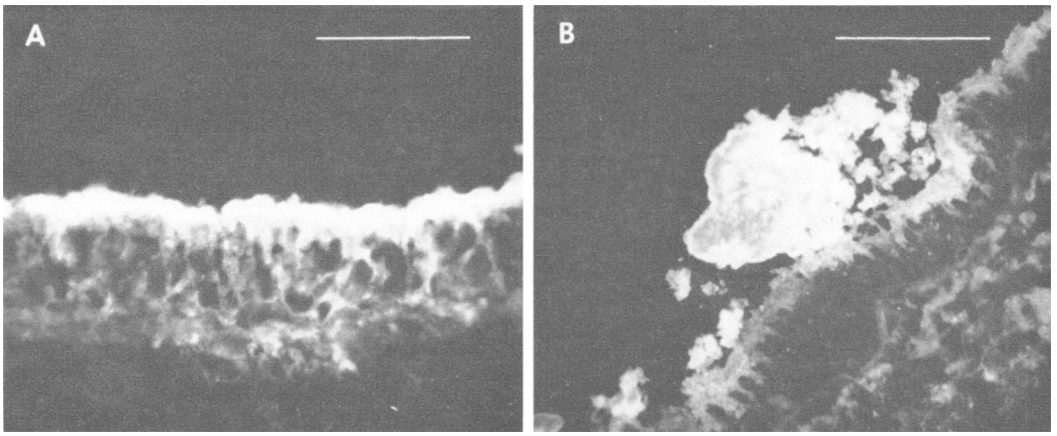


FIG. 1. Infected hamster tracheal organ culture reacted with *M. pneumoniae* antiserum: (A) 48 hr after inoculation; (B) 72 hr. (Indirect immunofluorescent technique; original magnification 640×; bars = 50 μ.)

By 48 hr, the ciliary action had slowed, and bright-staining granular material was noted along the luminal surface of the epithelial cells as demonstrated in Fig. 1A. Ciliostasis had occurred by 72 hr; large masses of specific antigen could be seen extending into the tracheal lumen (Fig. 1B), and abundant fluorescent material covered the epithelial surface. In addition, specific fluorescence was noted in the distal half of the epithelial cytoplasm, surrounding the unstained nuclei represented by small dark ovals in Fig. 1B. Similar changes were seen in 96-hr specimens but were less distinct due to destruction and loss of much of the epithelial cell layer.

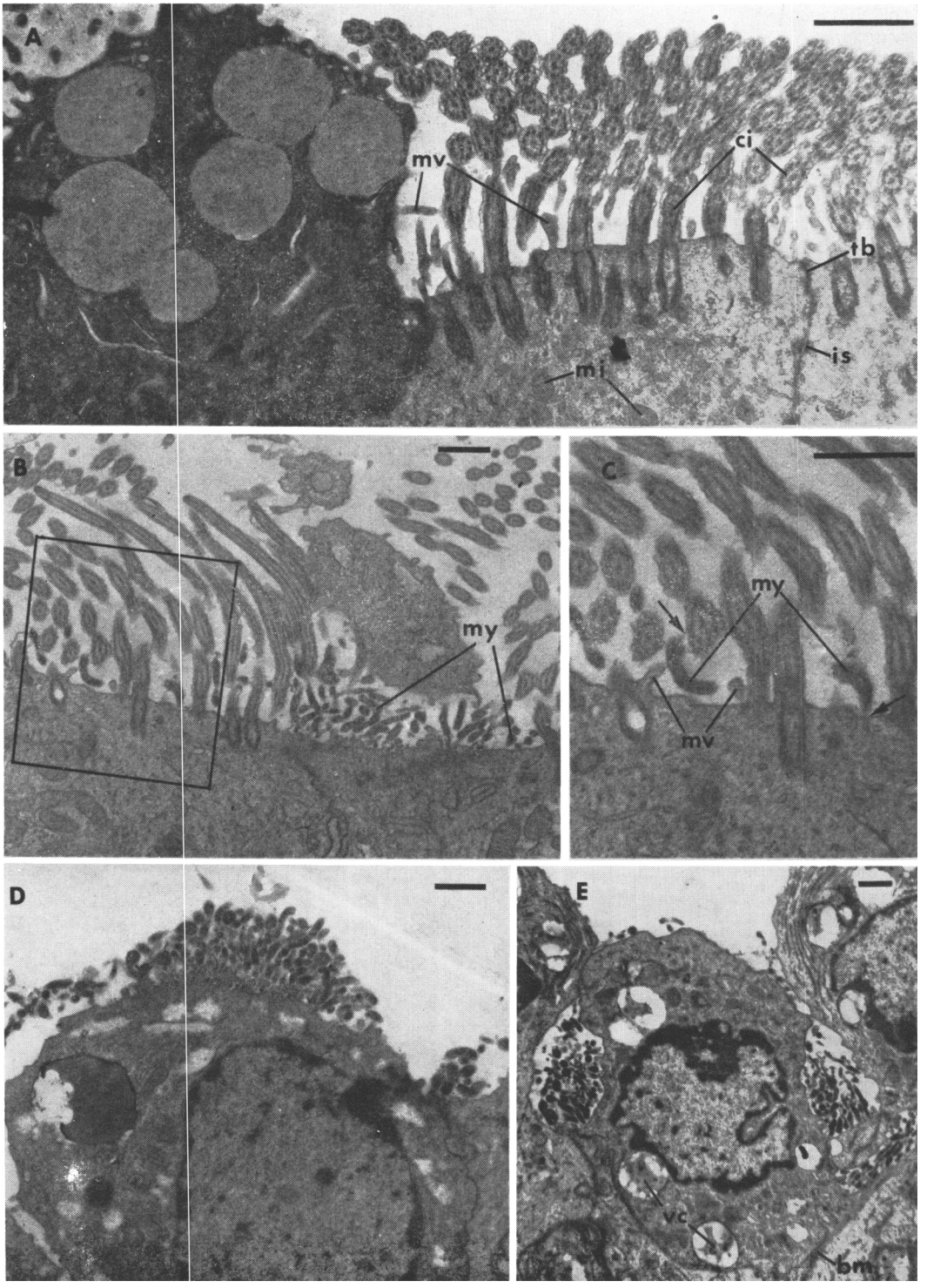
Control preparations included: (i) uninfected tissue reacted with *M. pneumoniae* antiserum and fluorescein-labeled antiglobulin; (ii) infected tissue reacted with normal rabbit serum and antiglobulin; and (iii) tissue stained only with the fluorescein-labeled reagent. No specific fluorescence was detected; all tissues showed faint gray-green cytoplasmic staining, and bluish autofluorescence of the epithelial basement membrane and cartilage.

The resolution permitted by the immunofluorescent technique was not adequate to define the relationship between individual organisms and epithelial cells. Since the majority of the epithelial cells appeared to be parasitized, it was felt that electron microscopy would be feasible for further study of this problem.

Electron microscopy. The ultrastructure of the hamster trachea has not been detailed in the literature, nor changes which may occur in organ culture. Preliminary study was made therefore of normal tracheas maintained in culture up to 72 hr. Detailed ultrastructure was preserved, as illustrated by Fig. 2A, which resembled findings reported for freshly removed rat trachea (6).

Infected tracheal sections which were processed for electron microscopy were compan-

FIG. 2. Electron photomicrographs of hamster tracheal organ cultures: (A) portions of a goblet cell (left) and ciliated epithelium (right) from uninfected tissue, showing cilia (ci), microvilli (mv), an intercellular space (is), a terminal bar (tb), and mitochondria (mi); (B) border of 3 epithelial cells infected for 48 hr; numerous mycoplasmas (my) are present at the base of the cilia; (C) enlargement of area outlined in (B). Arrows indicate points of attachment between organisms and cell membrane. (D) Microcolony of organisms on luminal border of cell infected for 72 hr; (E) invasion of intercellular spaces by organisms, and evidence of cell injury: (vc) vacuoles; (bm) basement membrane. (Original magnification 8000-24,000×; bars in each photograph = 1 μ.)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ions of those studied by immunofluorescence. A specimen 48 hr after inoculation with *M. pneumoniae* is illustrated in Fig. 2B and C. Numerous pleomorphic structures were found among the cilia, in correspondence to the distribution of specific antigen demonstrated in Fig. 1A. These structures were closely related to the epithelial cell membrane covering the base of the cilia, microvilli, and luminal border. The organisms were larger and more electron-dense than the microvilli, as shown by comparison of Fig. 2A and C. Figure 2C reveals presumptive points of attachment between organisms and the epithelial cell membrane.

By 74 hr after inoculation of the organ cultures (Fig. 2D), many cells showed loss of cilia and clumps of organisms were present on the luminal border, corresponding to the specific antigen demonstrated in Fig. 1B. In addition, microcolonies were now found in the intercellular spaces between adjacent epithelial cells, and between the epithelium and the basement membrane (Fig. 2). The terminal bars, which normally close the intercellular space at the epithelial border (Fig. 2A), were no longer seen. These extracellular organisms apparently account for the antigen surrounding cell nuclei demonstrated in Fig. 1B. A search of all sections failed to reveal organisms within the epithelial cells. In late specimens, ultrastructural evidence of cell injury also was demonstrated, including loss of cilia, numerous cytoplasmic vacuoles, and nuclear enlargement with clumping and margination of chromatin (Fig. 2D, E).

Discussion. Antigens of *M. pneumoniae* have been demonstrated in relation to a variety of host cell types by immunofluorescence. However, the small size of the organism has thwarted efforts to determine with conventional microscopy the exact site of cellular parasitism, an important factor in developing an understanding of pathogenetic mechanisms. The present investigations suggest, through correlation of immunofluorescence and electron microscopy, that the infection of differentiated epithelial cells is predominately if not exclusively extracellular.

Previous efforts to demonstrate *M. pneumoniae* in association with respiratory cells by electron microscopy have been unsuccessful. Donald and Liu studied infected chick embryos, and reported "elementary bodies of the virus" in the cytoplasm of nonciliated bronchial epithelium (7). However, since nothing was detected in ciliated cells which contained much of the antigen demonstrated by immunofluorescence, the implication of these findings is not clear. Lungs of gnotobiotic mice infected with *M. pneumoniae* were studied by Organick and Lutsky (8). These workers were unable to demonstrate the mycoplasma by electron microscopy, despite the presence of cultivable organisms and pneumonia in the lung tissue.

The difficulties experienced by both groups cited may have been due to technical nuances, or to the significant problem of adequate tissue sampling inherent in electron microscopy when an organ is examined. Use of tracheal organ cultures in the present studies offered the advantages of heavy and uniform infection of differentiated host cells in a controlled but isolated environment. The choice of fixatives and staining reagents clearly distinguished organisms from host cell constituents, which should facilitate further investigation of the relationships between these structures.

The nature of host cell parasitism by *M. pneumoniae* which was demonstrated in the present studies suggests several possible mechanisms of tissue injury. The intimate relationship of the organism and the epithelial cell membrane may allow either mechanical or chemical injury to occur. The masses of organisms among the cilia could interfere physically with normal beating, or epithelial membrane injury could result in disturbance of surface charges needed for synchrony of ciliary motion. Nutritional deprivation of host cells, by membrane injury or metabolic competition between host and parasite, could offer additional means of functional impediment. The organisms appeared able to break down the terminal bars, opening the intercellular space and disrupting the tissue architecture. These findings may have implications for further understanding of the pathophysiology of *M. pneumoniae* disease.

Summary. These studies illustrate the applicability of a tracheal organ culture system for the study of *Mycoplasma pneumoniae*

infection at the cellular level. The results of immunofluorescence and electron microscopy indicate an intimate association of *M. pneumoniae* and the ciliated epithelial cell membrane. While *M. pneumoniae* appeared able to break down terminal bars and enter the intercellular spaces no evidence of intracellular localization was seen. These findings provide additional insight into the pathogenicity of *M. pneumoniae*.

1. Collier, A. M., Clyde, W. A., Jr., and Denny, F. W., Proc. Soc. Exp. Biol. Med. **132**, 1153 (1969).
2. Hayflick, L., Tex. Rep. Biol. Med. **23**, 285 (1965).
3. Clyde, W. A., Jr., Denny, F. W., and Dingle, J. H., J. Clin. Invest. **40**, 1638 (1961).
4. Watson, M. C., J. Biophys. Biochem. Cytol. **4**, 475 (1958).
5. Millonig, G., J. Biophys. Biochem. Cytol. **11**, 736 (1961).
6. Rhodin, J., and Dalhamm, T., Z. Zellforsch. **44**, 345 (1956).
7. Donald, H. B., and Liu, C., Virology **9**, 20 (1959).
8. Organick, A. B., and Lutsky, I. I., J. Bacteriol. **95**, 2310 (1968).

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