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# *Mycoplasma pneumoniae* in Hamster Tracheal Organ Culture Studied by Scanning Electron Microscopy

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Hamster tracheal rings in organ culture were inoculated with a virulent strain of *Mycoplasma pneumoniae* and examined by scanning electron microscopy. A progressive increase in epithelial cell injury was detected from 48 to 96 h post-inoculation and was characterized by apparent loss of the apical portion of ciliated cells. *M. pneumoniae* attaching to the epithelial cell surfaces could be identified by comparison with the surface morphology of mycoplasmas grown on glass cover slips.

*Mycoplasma pneumoniae* is one of the more common etiological agents recovered from lower respiratory infections of children and young adults (13). Because of the low mortality associated with *M. pneumoniae* infections, much of the knowledge regarding disease pathogenesis has been provided by in vitro studies. Experimental *M. pneumonia* infections of hamster tracheal epithelium in organ culture have shown that the organisms attach noninvasively to the luminal border of the respiratory epithelial cells (10), with resultant inhibition of host cell protein and ribonucleic acid synthesis (15), alterations in cellular organelles (6), and cessation of ciliary activity (8). Transmission electron micrographs have been instrumental in demonstrating that *M. pneumoniae* adherence to ciliated epithelial cells occurs by a specialized terminal attachment organelle (8). As yet, however, knowledge is incomplete regarding the three-dimensional morphological aspects of the host cell-parasite interaction and resulting epithelial cell surface alterations.

The scanning elecron microscope (SEM) affords a unique high-resolution view of large surface areas of respiratory epithelium. With the SEM, investigators have demonstrated morphological alterations in ciliated respiratory epithelium after exposure to a variety of toxins (11), air pollutants (2), and infectious agents (24). In the present report, hamster tracheal rings in organ culture were infected with *M. pneumoniae* and studied in an SEM.

## MATERIALS AND METHODS

**Organisms.** The *M. pneumoniae* strain (M129) was originally isolated from a patient with pneumonia (18). The 10th Hayflick broth (14) passage of this isolate (M129-B10), which retained virulence as determined by its ability to produce pneumonia in Syrian hamsters after intranasal inoculation, was used in this study (12). *M. pneumoniae* in broth culture were prepared for growth onto glass cover slips (25 by 25 mm) by sonication and filtration through an 0.45-$\mu$m Millex filter (Millipore Corp., Bedford, Mass.) as previously described (23).

**Organ cultures.** Tracheal organ cultures were prepared from adult male, Syrian hamsters and maintained in Hayflick medium as described by Collier et al. (10). The tracheal rings were infected with *M. pneumoniae* as previously described (8, 10).

**Electron microscopy.** The glass-attached organisms and tracheal rings were washed with warm (37 C) phosphate-buffered saline and fixed in 2.5% glutaraldehyde in phosphate buffer at pH 7.4 for 1 h. The specimens were washed in 0.1 M phosphate buffer (4 C) for 12 h and dehydrated through graded ethanol solutions. After a transfer through a gradient of freon 113 in ethanol, the specimens were critical point dried with freon 13 in a Bomar SPC-50/EX apparatus. The samples were attached to specimen studs with Dag cement and subsequently coated with carbon and gold in a Denton vacuum evaporator. The specimens were viewed with an ETEC autoscan SEM operating at 10 to 20 kV. The images were recorded on Polaroid 55P/N film.

For transmission electron microscopy, tracheal rings were fixed as above in glutaraldehyde, washed in phosphate buffer, and postfixed in 1% osmic acid in 0.1 M phosphate buffer for 1 h at 4 C. The tissue was dehydrated in graded ethanol solutions and embedded in Epon 812 by the method of Luft (19). Thin sections were obtained with a Porter-Blum ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and examined in a Siemens 1A Elmskop electron microscope.

## RESULTS

**Scanning electron microscopy of *M. pneumoniae*.** During the initial 24 to 48 h of growth onto glass cover slips, *M. pneumoniae* were single or branching filamentous organisms with a bulblike "neck" and a tapered tip end

(Fig. 1). Individual mycoplasmas measured 190 to 220 nm in diameter and 2.2 μm in length and were interspersed with small aggregates of organisms, representing early colony forms. As surface growth progressed, these colony forms increased in diameter and height to globular configurations within which single mycoplasmas were no longer discernible. Individual *M.*

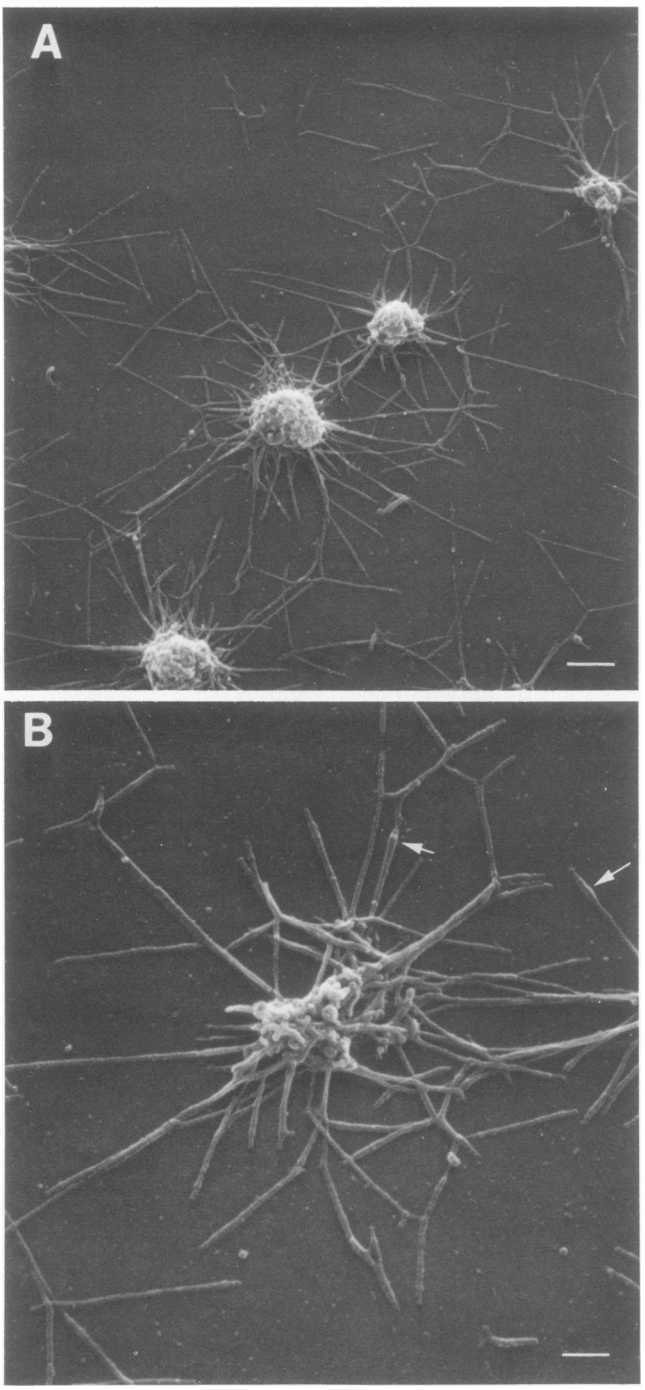


FIG. 1. *M. pneumoniae grown onto a glass cover slip for 48 h. (A) Note the uniform appearance of the filamentous organisms and the presence of a developing microcolony. Bar = 2 μm. (B) Individual mycoplasmas were characterized by a bulbous swelling adjacent to a tapered tip end (arrow). Bar = 1 μm.*

*pneumoniae* attached to the glass surface throughout their entire length, whereas in organ culture the attachment occurred primarily at the tip region with the body of the organism extending perpendicular to the epithelial surface (Fig. 2).

**Normal hamster tracheal epithelium.** Scanning electron microscopy of uninfected tracheal rings maintained in Hayflick medium for 24 h showed the luminal surface to be composed of two recognizable cell types (Fig. 3). The predominant cell possessed cilia measuring 4.5 to 5.0 μm in length and 300 nm in diameter interspersed with microvillus projections 150 nm in diameter and 200 to 600 nm in length. The nonciliated cells appeared dome shaped and were characterized by individual surface projections or microvilli 350 nm in diameter and 400 nm in length. Other than occasional thin strands, no mucous blanket was apparent on the luminal surface of tracheal rings that had been incubated in organ culture. This appearance contrasts with the dense mucous layer overlying hamster tracheal epithelium fixed in situ (20).

**Infected hamster tracheal epithelium.** Tracheal rings examined by SEM 24 and 48 h after inculation with *M. pneumoniae* showed loss of cilia when compared with uninfected controls. This reduction was accompanied by the appearance of extruded fragments of ciliated cells (Fig. 3) measuring 4 to 5 μm in diameter. These rounded extrusions were marked by a smooth-surfaced membrane and cilia showing a general lack of rigidity (Fig. 3D). Structures recognizable as surface-attached mycoplasmas were not observed at either 24 or 48 h of infection.

At 72 h post-inoculation, tracheal rings showed continued sloughing of ciliated fragments and a further reduction in the density of cilia. Cilia that remained attached appeared morphologically unaltered. The nonciliated surfaces appeared flattened with numerous knob-like microvillus projections measuring 150 to 170 nm in diameter. At this time period, *M. pneumoniae* adhering to the epithelial cell surfaces were readily identified by their thin filamentous morphology (Fig. 4A). Mycoplasmas were evenly distributed over ciliated cells at the base of cilia. Those accumulating on nonciliated surfaces were localized over the intercellular junctions with relative sparing of the central region of the cell (Fig. 4B). Even when densely aggregated, mycoplasmas adherent to mucosal cells maintained their individual filamentous appearance in contrast to the colony forms grown on glass.

At 96 h post-inoculation, only isolated tufts of cilia were evident on the luminal surface. The nonciliated surfaces, as at 72 h, remained characterized by the presence of microvillus projec-

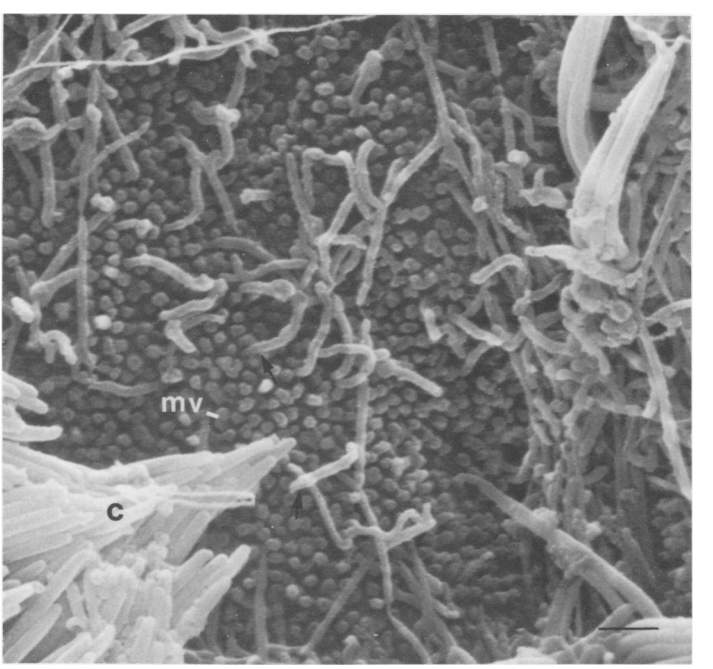


FIG. 2. *M. pneumoniae attached to hamster tracheal epithelium. Single organisms (arrows) attaching between microvilli are oriented perpendicular to the cell surface. c, Cilia; mv, microvilli. Bar = 1 μm.*


FIG. 3. *Hamster trachea in organ culture. (A) Uninfected control in culture for 24 h. (B) Trachea infected with M. pneumoniae for 24 h. (C, D) Extruding cell fragments (arrows) noted after 48 h of infection. Bar = 5 μm.*

tions. The cell surface was covered almost in its entirety by dense accumulations of *M. pneumoniae* (Fig. 5A). This increase in mycoplasmas appeared to occur as a result of centripetal growth of the organisms over the microvillus surface. Uninfected control rings at 96 h (Fig. 5B) were marked by the absence of surface-attached filamentous structures and good preservation of normal cellular surface features. Transmission electron micrographs of tracheal rings infected for 96 h (Fig. 6) confirmed the extensive accumulation of *M. pneumoniae* attached by its characteristic attachment structure (8) to the epithelial cell surface membrane.

## DISCUSSION

The in vitro model studied in this report has previously been examined by light (7), fluorescent (10), and transmission electron microscopy (7, 10) and has been utilized to clarify metabolic injury to epithelial cells after *M. pneumoniae* infection (15). The use of scanning electron microscopy in this study has provided further information regarding the host-parasite interaction and resultant host cell injury.

Because of conflicting reports (1, 16, 17) regarding the SEM appearance of *M. pneumoniae*, initial studies were made of glass-at-

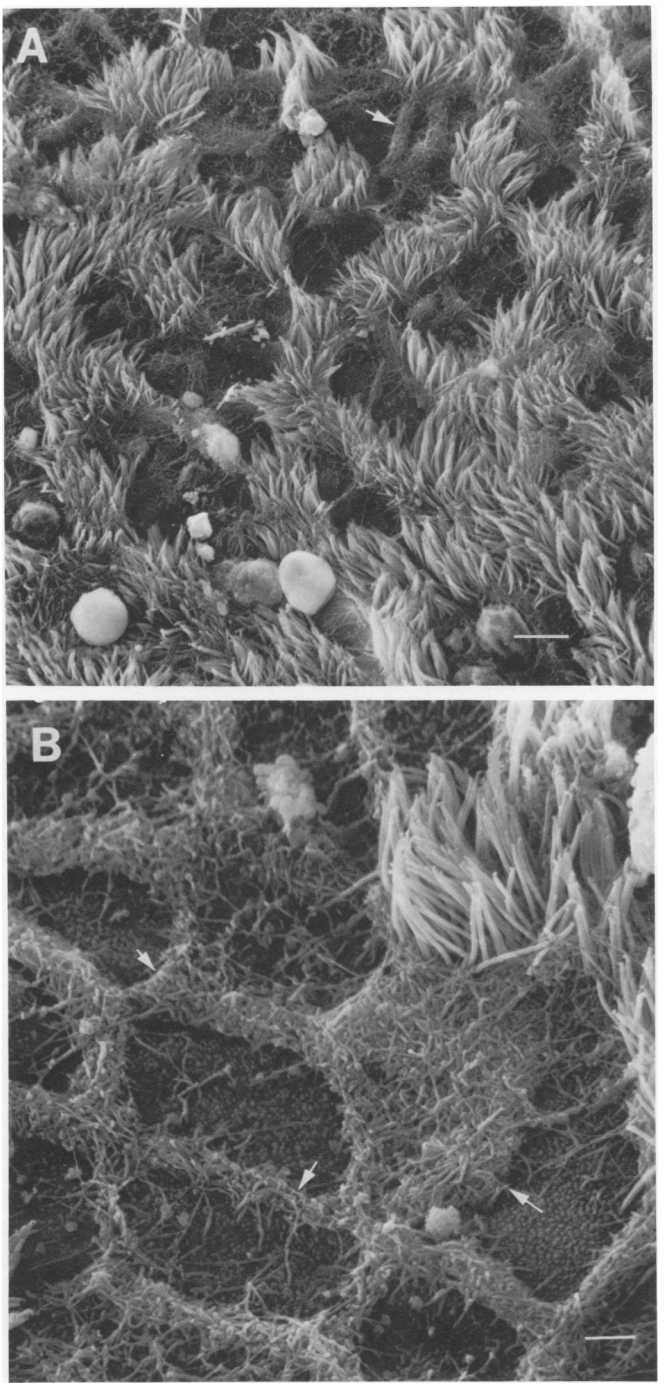


FIG. 4. *Hamster trachea in organ culture infected with virulent M. pneumoniae for 72 h. (A) Note the apparent loss of cilia and the recognition of surface-attached organisms. Bar = 5 μm. (B) M. pneumoniae localized along the intercellular boundaries (arrow). Bar = 2 μm.*

tached organisms. Our findings agree with those of Biberfeld and Biberfeld (1) with the additional notation of a consistent "bulblike neck" terminating in a tapered tip. This morphology does not appear to be artifactual and has been described in phase microscopic studies of glass-attached *M. pneumoniae* (3). Bredt (4), by using phase microscopy, has shown that the

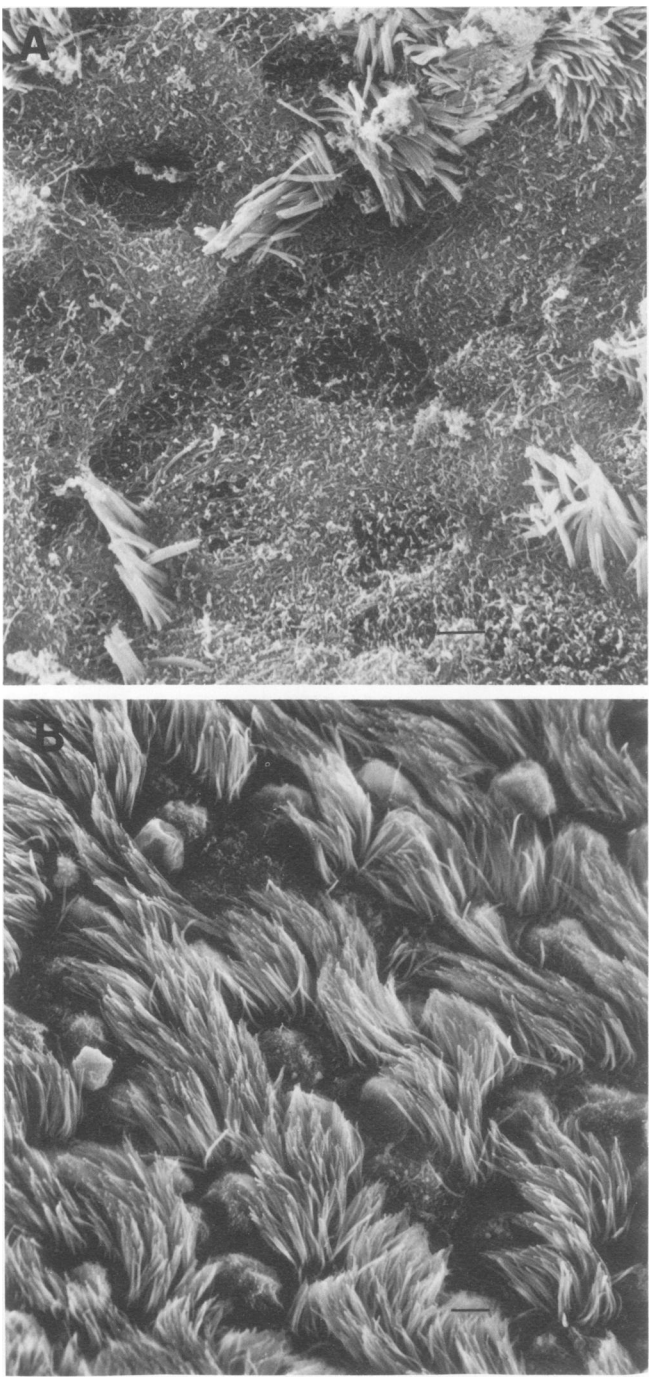


FIG. 5. *Tracheal epithelium after 96 h in culture. (A) Trachea infected with M. pneumonia. Bar = 3 μm. (B) Uninfected trachea. Bar = 5 μm.*

tip is the primary attachment point of *M. pneumoniae* to glass surfaces and is the lead point in directional changes by this motile pathogen.

In SEM examination of organisms attached to tracheal epithelial cells, this tip structure was less well discerned. However it was apparent that individual mycoplasmas attached to these cells with one end and were oriented per-

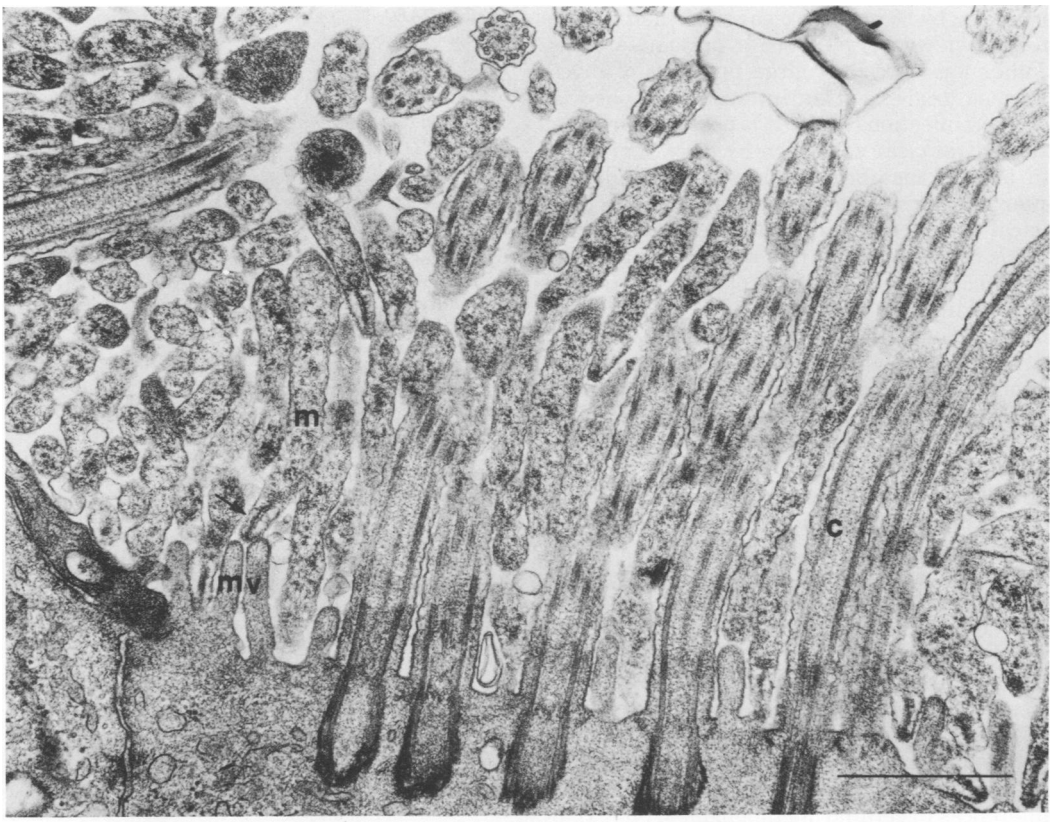


FIG. 6. *Transmission electron photomicrograph of tracheal ciliated cell infected with M. pneumoniae for 96 h. The arrow denotes the characteristic attachment structure of M. pneumoniae. c, Cilium; m, mycoplasma; mv, microvillus. Bar = 1.0 μm.*

pendicular to the cell surface. Furthermore, the initial contact with the cell membrane appeared to involve primarily single organisms; only at later stages of infection were masses of filamentous mycoplasmas noted. A recent transmission electron micrograph study of human sputum obtained during the acute stages of *M. pneumoniae* infection (9) has shown heavy parasitization of extruded epithelial cells by filamentous *M. pneumoniae*. This finding, coupled with the present study, suggests that the infective form of *M. pneumoniae* is a single, filamentous organism rather than the amorphous colony forms that predominate in late stages of *M. pneumoniae* growth onto glass surfaces or in broth culture (1).

In the present study, specific epithelical cell alterations in tracheal rings infected with *M. pneumoniae* became apparent by 48 h with the observation of exfoliating ciliary tufts. The rounded appearance and small size that characterized these extrusions suggest they represent only the apical portion of ciliated cells. This observation is consistent with previous transmission electron micrograph descriptions of *M. pneumoniae*-infected tracheal rings in which a ballooning degeneration or "sunburst" appearance of the apical region of the ciliated cells is apparent (6). The observation made with SEM suggest that this ballooning apical portion is ultimately sloughed from the main body of the cell. Such fragmentation of ciliated cells has been described by Papanicolaou (21) and termed ciliocytophoria. Pierce and Hirsch (22), studying sputum samples of adults during acute respiratory disease, found ciliocytophoria in nearly all sputum samples from patients with influenza pneumonia, cold agglutinin-positive, atypical pneumonia, or viral pneumonia of undetermined etiology. Carilli and co-workers (5) have described ciliocytophoria in the sputum from two patients with proven *M. pneumoniae* infections.

By 72 h post-inoculation, many nonciliated tracheal epithelial cells contained a central clear zone and accumulations of *M. pneumoniae* over the intercellular junctions. It seems unlikely that this distribution of mycoplasmas resulted from preferential attachment of growth of *M. pneumoniae* along the cell periph-

ery since, by 96 h, these clear zones had become overgrown with organisms. In previous studies, Collier has described large numbers of mycoplasmas located within intercellular spaces at 72 h postinfection (7, 10). It is possible then that the central clear zone seen by SEM represents an area of membrane surface surrounded by intercellular mycoplasmas remaining after the apical portion of the cell has been sloughed.

The SEM appearance of cellular injury after *M. pneumoniae* infection contrasts with patterns of injury described in other SEM studies of tracheal epithelium. Reed and Boyde (24) infected bovine trachea in organ culture with a rhinovirus and noted that entire cells were extruded from the luminal surface, often maintaining a thin cytoplasmic attachment to the basement membrane. Dahlgren et al. (11), studying acrolein-induced epithelial injury in guinea pig trachea, found a reduction in density of cilia accompanied by a great variation in individual ciliary length, indicating selective ciliary damage. No hint of such ciliary injury was detected in the *M. pneumoniae*-infected cells.

The contrasting appearances of damaged tracheal mucosa demonstrate the usefulness of SEM in differentiating patterns of cellular injury and suggest that these morphological differences may be associated with identifiable differences in cellular metabolism. *M. pneumoniae* infection of organ culture provides the opportunity to make correlations of this type. Hu et al. (15), using the same in vitro model as studied in this report, have demonstrated decreased ribonucleic acid and protein synthesis by epithelial cells infected with *M. pneumoniae* for 24 to 48 h. It would appear that the maximum exfoliation of ciliary tufts as seen with SEM occurs soon after this decrease in ribonucleic acid and protein synthesis. The association of a decrease in macromolecular synthesis with the loss of the apical portion of the cell is at present unclear. By 96 h, in the studies of Hu et al., marked decreases in orotic acid, amino acid, and galactose transport into the cells were seen. From the SEM studies, it is apparent that this represents a time period when there are few remaining cilia and the luminal surface is literally coated with mycoplasmas. This suggests that decreased precursor transport might represent either a loss of transport sites associated with the apical portion of the ciliated cells or a steric block of available transport sites by attached organisms.

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