

Cytopathogenicity of *Mycoplasma hyopneumoniae* in Porcine Tracheal Ring and Lung Explant Organ Cultures Alone and in Combination with Monolayer Cultures of Fetal Lung Fibroblasts

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Porcine tracheal rings and lung explants alone and in combination with monolayer cultures of porcine lung fibroblasts (PLF) were separately inoculated with virulent strains of *Mycoplasma hyopneumoniae* and incubated at various times. The preparations were observed by bright-field, phase-contrast, and scanning electron microscopy. In PLF cultures, the strains at initial concentrations of $10^{1.3}$ colony-forming units/ml increased within 3 days to 10^6 colony-forming units/ml, showed progressive clustering on the cells, and caused some sloughing. Introduction of a tracheal ring or lung explant into these mycoplasma-infected PLF cultures caused the explant to lose its epithelial ciliary motility. Eventually parts or whole cells of the respective ciliated epithelium were lost. Without infected PLF monolayers, the explants inoculated with *M. hyopneumoniae* were less susceptible to infection. When uninfected explants were incubated for 18 days or kept in stock for 2 months, they did not show the above changes. Within 5 h postinoculation, *M. hyopneumoniae* cultures became intimately associated with the PLF culture, but when epithelial cell sloughing occurred, the mycoplasmal cells became dependent on the introduction of a fresh PLF monolayer or a tracheal or lung explant for survival.

Mycoplasmas spp. are associated with a variety of diseases in animals (1). In swine, the primary cause of pneumonia is *Mycoplasma hyopneumoniae* (15). Clinical features of the disease were characterized by Betts (2), and many investigators have later concurred with his description of the disease (15). Electron microscopic examination of lung tissues removed from infected pigs over an extended period of time revealed mycoplasma-appearing material in close proximity to cilia of bronchial epithelial cells along with decreased numbers of cilia (11). However, further characterization of various degenerative stages of the disease is needed because secondary infections with bacteria, viruses, other *Mycoplasmas* or nematodes commonly occurring in the host in field cases of the disease may alter the tissue response and result in more severe morphological changes (14).

Because *M. hyopneumoniae* primarily interacts with the host on the ciliated epithelial surfaces of the respiratory system, further study is indicated (11). The investigation of this infection in porcine respiratory tissue cultures by scanning electron microscopy affords a unique high-resolution view of surface areas of the tissues which

could be compared with other microscopic observations. These techniques could facilitate an assessment of the pathogenicity. The present report is concerned with growth and parasitic modes of *M. hyopneumoniae* in porcine respiratory organ and monolayer cultures maintained in a medium supporting tissue growth which does not support growth of the organism in the absence of tissue.

MATERIALS AND METHODS

Organisms. Strains of *M. hyopneumoniae* used were ATCC 25617, NU-16, B245 (C. L'Ecuyer, Ph.D. thesis, Iowa State University, Ames, 1962; C. W. Livingston, Jr., Ph.D. thesis, University of Nebraska, Lincoln, 1970; C. J. Maré, Ph.D. thesis, Iowa State University, Ames, 1965; all obtained from D. O. Farrington, Veterinary Medicine Research Institute, Iowa State University, Ames), and NADC-2069. Virulence of these strains was shown by transtracheally inoculating 4- to 8-week-old hysterectomy-derived pigs and observing for production of pneumonic lung lesions (Williams, unpublished data).

Media. Strains of *M. hyopneumoniae* were cultured on a modified Friis medium with and without agar (6).

Organ cultures. Tracheal ring (TR) and lung (L) explant organ cultures 0.5 to 1.0 mm thick were pre-

pared from fetal porcine respiratory systems as described previously (19). Each TR or L explant was incubated at 37°C in the window of a rubber-stoppered Leighton (16 by 125 mm) tube. The tube was rotated (12 rph) to expose the tissues to alternating gaseous and medium phases. Filter-sterilized Eagle minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 20% (vol/vol) fetal calf serum, sodium bicarbonate (2.2 mg/ml), sodium pyruvate (10 mg/ml), lactalbumin hydrolysate (5 mg/ml), benzylpenicillin potassium (100 U/ml), and streptomycin (sesquisulfate) (100 µg/ml) at pH 7.4 served as culture support medium (CSM).

Each TR or L culture was replenished with 1.5 ml of CSM per tube every 3 days. On day 6 of incubation, the TR and L cultures were each selected for experimentation on the basis of active and uniform ciliary motility and cellular integrity. The selected TR and L cultures were then randomly chosen in duplicate for use as uninoculated (uninfected) controls and experimentals inoculated with *M. hyopneumoniae*. Each preparation was placed in 1.5 ml of fresh CSM in the window of a Leighton tube with or without monolayer cultures of porcine lung fibroblasts (PLF) grown on a coverslip (11 by 35 mm). Each tube was then inoculated with 0.5 ml of CSM or 0.5 ml of *M. hyopneumoniae* obtained from four consecutive, 6-day, 37°C incubation passages in four corresponding 1.5-ml-PLF monolayer cultures in CSM, or 0.5 ml of a 6-day-old modified Friis broth culture of *M. hyopneumoniae*. Duplicate preparations were removed at 0, 5, 10, and 24 h and at 3, 6, 9, 12, and 18 days of incubation. Every 3 days, CSM was removed by aspiration and replaced with fresh CSM. The PLF monolayers were removed and replaced every 6 days where indicated (Table 1). PLFs were heat inactivated at a temperature of 56°C for 30 min. All explants were observed for ciliary movement by use of an inverted-light microscope. Tissues were examined by scanning electron microscopy and for histopathology. The above experiment was repeated in tests to determine *M. hyopneumoniae* titers during the incubation periods.

Sterility tests (thioglycolate broth and blood agar plates) were conducted periodically during these experiments. The cultures were also examined for contaminating wild mycoplasma strains in modified Friis broth medium. The broth medium was subcultured to agar medium after 4 to 7 days of incubation, and the latter was examined for colonies after 1 week.

Tissue culture. Primary cultures of fetal PLF were prepared by standard methods (5, 20), CSM was used as a growth medium in tissue culture flasks. Subcultures of the monolayers were dispensed into Leighton tubes and grown on coverslips (11 by 35 mm) covered with CSM.

Histopathology. Tissue samples were removed at the above incubation intervals, fixed in 10% buffered neutral Formalin, and embedded in paraffin. Tissue sections 5 to 7 µm thick were cut and stained with hematoxylin and eosin.

Electron microscopy. Explants of TR and L were washed three times with 0.1 M phosphate-buffered saline, pH 7.2, and fixed in 3.0% paraformaldehyde in phosphate-buffered saline for 1 h at room temperature. The specimens were washed in phosphate-buffered

saline for 12 h and dehydrated through 25, 25 to 50, 75, 85, and 95% and absolute (four times) acetone solutions at 15-min intervals. The specimens were dried in Freon 13 (monochlorotrifluoromethane) and attached to aluminum-mounting specimen stubs with silver-primed conductive paint. The treated specimens were coated with gold to a thickness of ≤40 nm in a vacuum evaporator. The coated samples at a stub angle of 45° were examined in a Stereoscan scanning electron microscope operated at 20 kV. The images were recorded on Polaroid positive-negative type 105 film.

RESULTS

Porcine TR and L epithelial surfaces. Uninfected (control) TR and L cultures showed luminal surfaces with folds comprised of homogeneous mosaics of ciliated respiratory epithelial cells (Fig. 1 and 2). Cilia from 4.5 to 5.0 µm long were uniformly positioned on the epithelial cells. These cilia were slightly curved and arranged parallel to each other (Fig. 3). Occasionally non-ciliated areas were observed with microvilli on the apical regions of goblet cells (Fig. 4). The organ cultures, with and without PLF monolayers, retained their cellular differentiations and ciliary motilities and showed no buildup of debris in airway passages. Cellular overgrowths and disruption of cellular structures were absent (Table 1, groups A and B).

Infected organ cultures without monolayer cultures of PLF. By direct microscopic examinations, *M. hyopneumoniae* strain ATCC 25617 in TR and L explants without PLF monolayers showed no ciliostasis in the first 6 days of incubation, even though the mycoplasma titers increased from 10^{1.3} to 10⁶ colony-forming units/ml in the first 3 days. By days 9 and 12, infected TR and L cultures showed some ciliostasis without apparent tissue damage. By day 18, infected TR and L cultures showed extensive ciliostasis and some exfoliation of cilia (Table 1, group C). *M. hyopneumoniae* strain ATCC 25617 titers at this time were 10⁶ to 10⁷ colony-forming units/ml. Histological sections of the TR and L tissues showed no basement membrane or lamina propria cellular disruptions. Coccoid-to-bacillary, mycoplasma-appearing particles on the TR and L luminal surfaces were occasionally observed in scanning electron microscope scans.

Infected organ cultures with monolayer cultures of PLF. When fresh PLF monolayers were added to TR or L cultures, strain ATCC 25617 increased in mycoplasma cell concentrations for the first 3 days. The highest titers observed ranged from 10⁶ to 10⁷ colony-forming units/ml. Some cilia in TR and L cultures at 3 days were nonmotile until fresh CSM was added

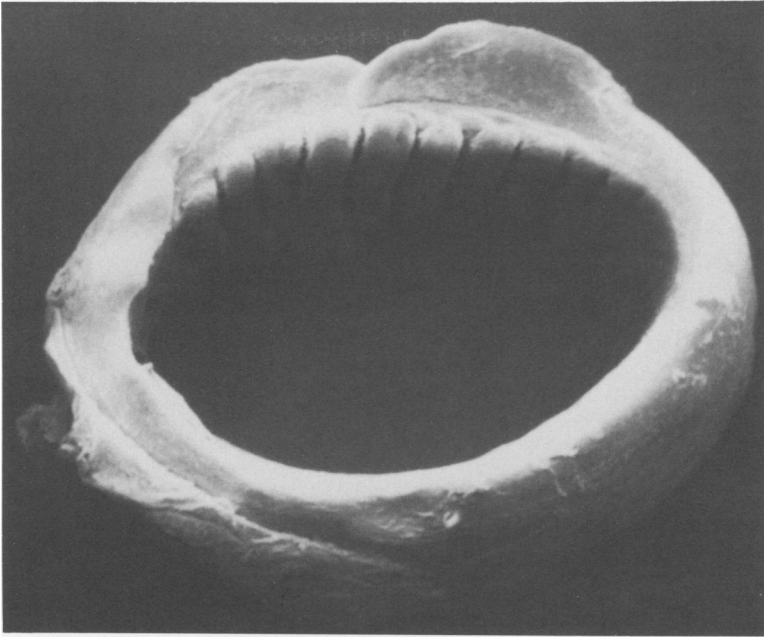


FIG. 1. *Scanning electron photomicrograph of a tracheal ring after 1 day in culture with monolayer cultures of PLFs. The ring is uniform in thickness at all points of its circumference, except at the cartilage-free area corresponding to the dorsal surface of the trachea. There is no debris or disruption of tissues, but loose adventitia tissue is present on the outer surface of the ring; ×21. Migrating cells blended with those at the cut margins and provided healed, rounded surfaces to the ring within 3 days.*



FIG. 2. *Scanning electron photomicrograph of a lung explant culture incubated for 6 days with monolayer cultures of PLFs. A bronchiole is shown with many ciliated epithelial folds. The tissues remained in a differentiated state in culture and showed conformities structurally similar to those observed in freshly cut preparations; ×100.*

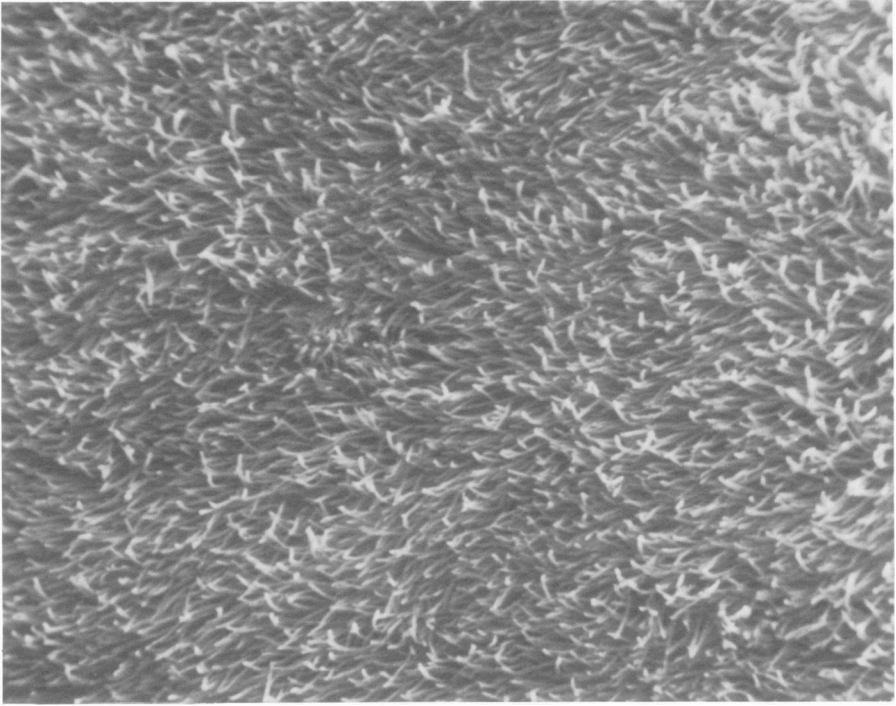


FIG. 3. Scanning electron photomicrograph of a control uninfected tracheal ring culture incubated for 24 days. The luminal epithelial cells are uniformly ciliated, and the cilia appear to be similar in length and diameter. The slightly curved cilia are arranged parallel to each other and lean in the direction of the larynx in situ; $\times 2,000$.

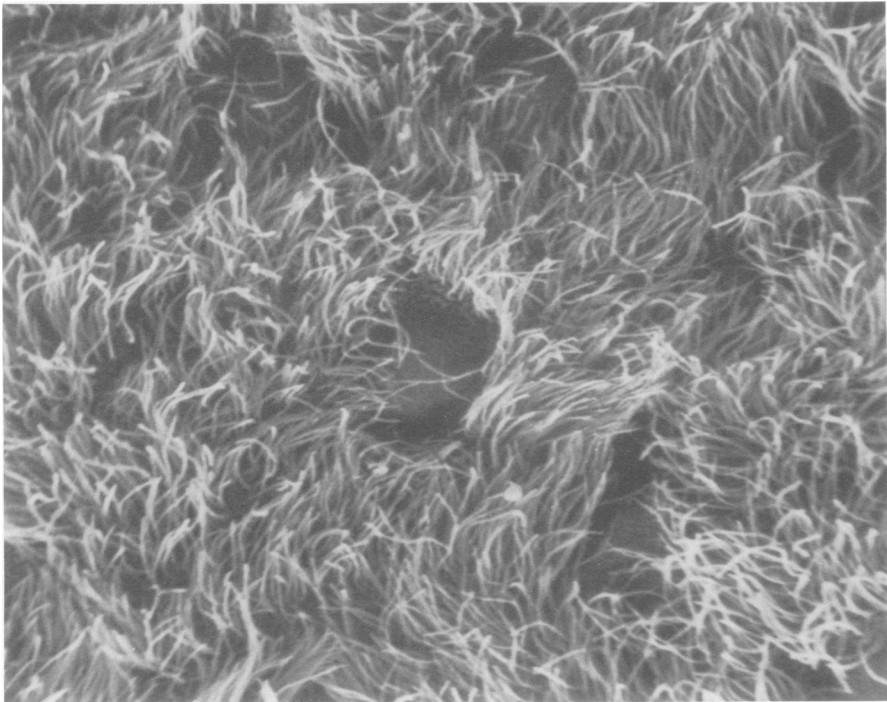


FIG. 4. Scanning electron photomicrograph of a control uninfected bronchiolar region of a lung explant incubated for 24 days. The luminal epithelial cells are mostly ciliated, and the cilia appear to be similar in length and diameter. Microvilli on the apical regions of goblet cells are detectable in the center of the photograph; $\times 2,100$.

TABLE 1. *M. hyopneumoniae* strain ATCC 25617 epithelial damage in porcine TR and L explant cultures alone and in combination with monolayer cultures of PLF

Group	Organ culture ^a	Mean score of epithelial ciliary motility and tissue conditions corresponding to days incubated at 37°C postinoculation ^b					
		0	3	6	9	12	18
A. Uninoculated control	TR	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N
	L	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N
B. Uninoculated control with uninoculated PLF monolayers	TR	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N
	L	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N	1.0, N
C. <i>M. hyopneumoniae</i> -inoculated organ cultures without PLF monolayers	TR	1.0, N	1.0, N	1.0, N	1.0, C	1.3, C	1.5, Ex
	L	1.0, N	1.0, N	1.0, N	1.0, C	1.3, C	1.5, Ex
D. Organ cultures with <i>M. Hyopneumoniae</i> -infected PLF monolayers	TR	1.0, N	1.3, N	1.5, C	1.8, Ex	2.5, Ex	3.0, SEC
	L	1.0, N	1.3, N	1.5, C	1.8, C	2.3, Ex	3.0, SEC
E. Organ cultures with <i>M. hyopneumoniae</i> in heat-inactivated (56°C, 30 min) PLF monolayers	TR	1.0, N	1.0, N	1.0, N	1.3, C	1.3, C	1.5, Ex
	L	1.0, N	1.0, N	1.0, N	1.3, C	1.3, C	1.5, Ex
F. Heat-inactivated PLF monolayers	NT	1.8	2.5	3.0	3.0	3.0	3.0
G. Uninfected PLF monolayers	NT	1.0	1.0	1.0	1.0	1.0	1.0
H. <i>M. hyopneumoniae</i> -infected PLF monolayers	NT	1.0	1.3	1.5	1.5	1.8	1.8

^a CSM was exchanged every 3 days. PLFs were replaced every 6 days. NT, Organ cultures not tested.

^b Based on arbitrary scale of 1 to 4 (19); 1 indicates a normal appearance and 2, 3, or 4 indicates progressively more severe damage. Mean scores were based on four replicates per incubation interval. N, Normal activity; C, ciliostasis; Ex, exfoliation of cilia and presence of nonmotile ciliary tufts; SEC, sloughing of epithelial cells.

and the preparations were incubated (Table 1, group D). From days 6 to 18 of incubation, both TR and L explants infected with *M. hyopneumoniae* showed similar progressive epithelial ciliostasis and cilia exfoliations (Table 1, group D). Remaining tufts of cilia occasionally were found that continued to beat. Some of these cilia showed loss of their parallel arrangements and gentle curvature and appeared to have a tendency to adhere to one another and, in extreme cases, to become matted. *M. hyopneumoniae* causes either TR or L cilia to undergo a straightening or bending, or both, before their exfoliation (Fig. 5). Prolonged incubations of TR and L cultures gradually deteriorated the luminal surface, with some loss of epithelial cells (Fig. 6). In histological preparations, small smooth-surfaced aggregates of ovoid-to-rounded bodies were observed on the TR and L luminal surfaces and in the deep recesses between cilia in lobar and proximal bronchioles. These mycoplasma-appearing bodies varied in size from approximately the same diameter as the cilium to several times larger. Sometimes these rounded bodies appeared in close contact with the epithelium and the bases of cilia (Fig. 6).

Comparisons of other *M. hyopneumoniae* strains with strain ATCC 25617 showed that strains NU-16, B245, and NADC-2069 were similar in virulence. No strain showed sustained growth in CSM in the absence of a TR or L explant or a PLF monolayer.

Histological observations of TR and L explants. In TR and L cultures of uninfected (control) cultures, the lumens were uniformly lined with ciliated pseudostratified columnar epithelial cells. In the larger airways (>700 μ m in diameter), a few goblet cells were present, and cell surfaces were free of cilia. Tissues lining the lobar and proximal bronchioles (\leq 700 μ m in diameter) consisted of many ciliated and some nonciliated areas with microvilli.

In the infected TR and L tissues, all lesions were limited to the epithelial layer and were seen on day 18 of incubation (Table 1, group D). Some of the epithelial cells of both TR and L cultures were free of cilia and sometimes appeared swollen, whereas others had lost some of their cellular materials (ciliocytophoria). In extreme cases, some of the epithelial cells free of cilia sloughed from the basement membranes. At no time did any of the preparations show disruption of the basement membranes or alteration and impairment of the lamina propria cells. *M. hyopneumoniae* strains NU-16, B245, and NADC-2069 showed cytopathological changes comparable to those observed for strain ATCC 25617.

Infected PLF. As early as 5 h, PLF monolayers inoculated with *M. hyopneumoniae* showed single coccoid organisms and clusters of mycoplasma-appearing materials in close proximity with the cell sheet. Some damage to the cell sheet was observed after 3 days of incubation

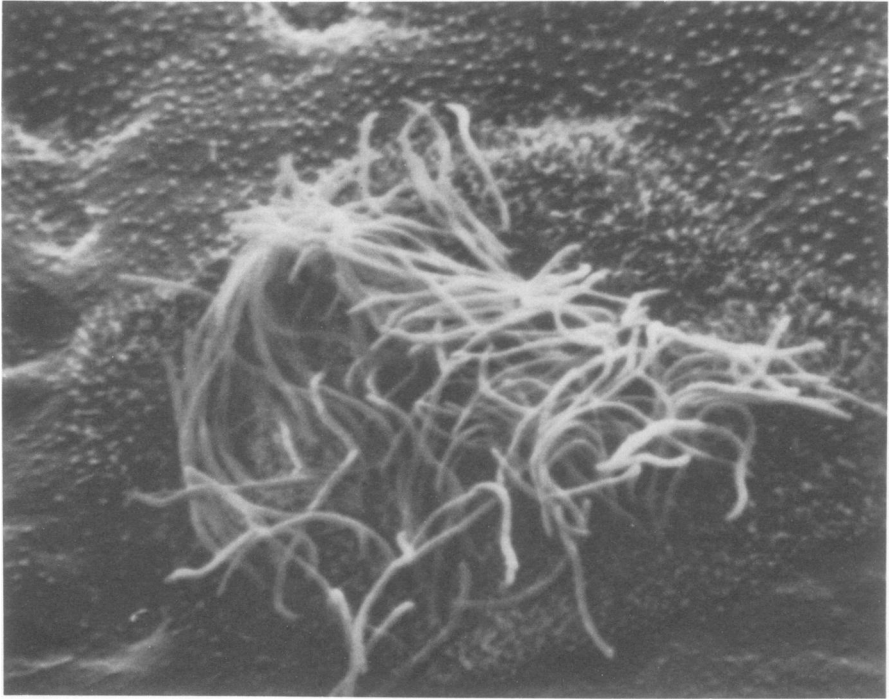


FIG. 5. Scanning electron photomicrograph of a bronchiolar region of a lung explant infected with *M. hyopneumoniae* strain ATCC 25617 incubated for 12 days postinoculation in the presence of monolayer cultures of PLFs. The luminal epithelial cells have lost their cilia. Remaining tufts of bronchiolar cilia are surrounded by microvilli on the surface. Cilia have lost their parallel arrangement and gentle curvature; $\times 5,400$.

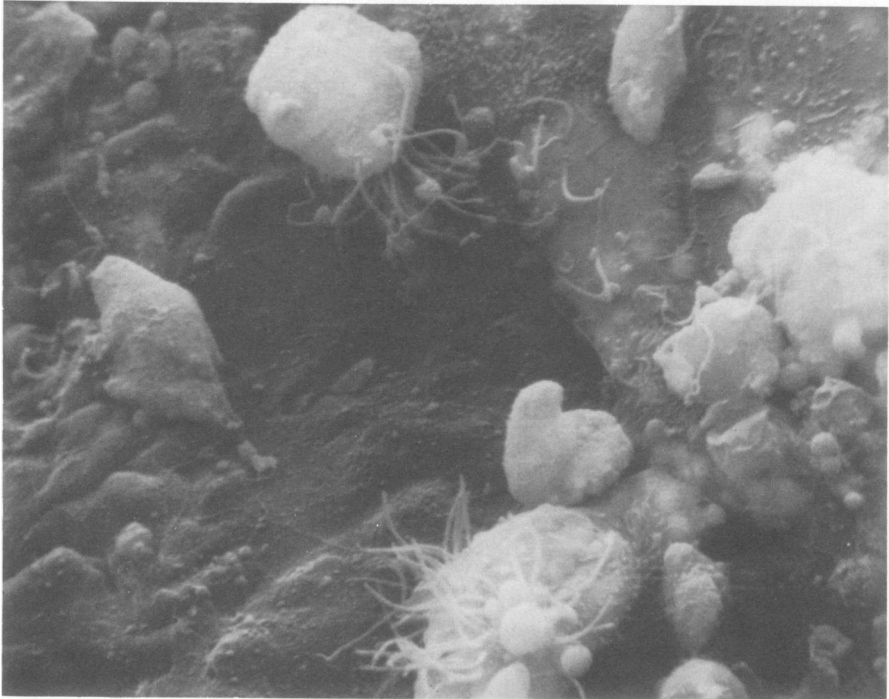


FIG. 6. Scanning electron photomicrograph of a lung explant infected with *M. hyopneumoniae* strain ATCC 25617 incubated for 18 days postinoculation in the presence of a monolayer culture of PLF. Small aggregates of mycoplasma-appearing materials are present, with deteriorating epithelial cells possessing a few remaining straightened or bent cilia, or both, that may have some mycoplasmal cells attached next to the luminal surface; $\times 2,300$.

(Table 1, groups G and H). Small coccoid organisms deeply stained as single particles or in clusters were on the exposed glass surfaces and on some of the epithelial cells attached to the glass substratum. Monolayers of PLFs introduced on day 3 showed increased mycoplasmal damage to the PLF monolayers on day 6 of incubation. Small, deeply stained coccoid organisms and "ring" forms were observed on the sloughed cells. Some of the attached epithelial cells in the remaining monolayer had long, thin cytoplasmic processes that sometimes showed clusters of coccoid organisms. On days 9, 12, and 18, observations were similar to those seen on day 6. In addition, on days 12 and 18 cells showed deeper stained nuclei, and the cytoplasm was vacuolated and more granular in appearance than the controls.

Titers of *M. hyopneumoniae* were between 10^6 to 10^7 colony-forming units/ml from days 3 to 18 of incubation. The uninfected controls through all incubation intervals showed intact cell sheets, and cells showed none of the cytopathological observations described above for infected PLFs.

DISCUSSION

Ciliated pseudostratified columnar epithelial cells of the trachea and lobar and proximal bronchioles, combined with mucous secretions and alveolar macrophages, play important roles in removing various inhaled particles and disease-promoting organisms from the respiratory system (1, 8, 17). Normal cilia of the tracheobronchial tree have a gentle curvature and lean toward the larynx (8), which is taken as evidence of directional ciliary beating for facilitating airway clearance. If these cilia were disrupted, then efficiency in clearing the airways would be impaired. Therefore, as noted by its cytopathological effects, *M. hyopneumoniae* in this regard would be detrimental to the host. This organism is unique in that it is the only agent that, when inoculated into pigs, will produce porcine enzootic pneumonia (15).

The results indicate that a relatively low inoculum of *M. hyopneumoniae* free of other microorganisms causes in vitro focal damages to tissues of the porcine respiratory system. The organism probably induces loss of cilia through elaboration of an enzyme(s) or toxic substance (7, 16). Once cilia have been lost, the organism could localize as a result of the diminished protective movement of the mucous blanket and allow adhesion to the fixed parts of the respiratory cells (4). In organ cultures, *M. hyopneumoniae* had no preference for infecting either TR epithelial cells or L epithelial cells.

Medium conditions appear to be important, because when additional cells (PLFs) were present, thus causing further use of medium constituents and buildup of metabolic end products, *M. hyopneumoniae* showed greater damage. It was difficult to determine whether *M. hyopneumoniae* cells infiltrated the PLF monolayers or the TR and L explants, since its morphological characteristics resemble particulate materials in tissue culture (3, 4; P. P. Williams, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, G7, p. 131). We could not determine whether the strains multiplied intracellularly or extracellularly, but the fact that it was dependent upon invasion of, or close contact with, the epithelial cells, or upon the presence of cell products, was shown by the inability of the strains to grow in CSM alone.

Cytopathological effects of *M. hyopneumoniae* with porcine TR and L cultures resemble those of the human respiratory pathogen *Mycoplasma pneumoniae* with hamster tracheal organ cultures (7, 10). At present, we do not specifically know how *M. hyopneumoniae* causes cytopathological effects. The organisms may cause an effect by producing a toxic substance, by parasitizing and destroying the cells, or by competing with the organ cultures for nutritional materials. It appears unlikely that these effects were due to release of PLF cellular constituents or to medium fluctuations in pH and NaCl and serum concentrations, since we have found TR and L explants to be relatively resistant to culture conditions (17, 19). However, cilia of the explants are quite sensitive to redox potentials (Williams, unpublished data). With some strains of *M. hyopneumoniae*, no apparent cytopathological effects occur in porcine TR cultures and in porcine kidney primary tissue cultures (12, 13). In in vivo situations, *M. hyopneumoniae* causes specific damage to the respiratory ciliated epithelial cells by decreasing the numbers of cilia (11). Virulent strains of *M. hyopneumoniae* used in our investigation have been recently shown to cause pulmonary lesions in hysterectomy-derived pigs, and all strains appear to be immunologically similar (Williams, unpublished data).

The development of techniques for the maintenance of TR and L organ cultures for ≥ 2 months (19) in order to study mycoplasma-induced disease processes results in obvious advantages over in vivo systems and should lead to their widespread use, such as for the isolation and growth of various respiratory tract viral, bacterial, and yeast pathogens (9, 18). Fully differentiated cells are present in organ cultures, which may vary widely in their susceptibility to mycoplasmal infections. The fact that these cells

are not in contact with immune cellular responses implies that the complexity of a given infection falls far short of that which occurs in vivo. Therefore, we would not be surprised if a particular infection in organ culture showed features that are unlike either tissue culture or in vivo infections but that may nevertheless bear on the mechanisms operating in vivo.

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