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Exfoliation of Respiratory Epithelium in Hamster Tracheal Organ Cultures Infected with *Mycoplasma pneumoniae*

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Summary. Hamster tracheal organ cultures were infected with *M. pneumoniae* and examined sequentially by transmission and scanning electron microscopy to correlate surface with intracellular alterations. Infection was established by culture and the demonstration of morphologically compatible organisms on the mucosal surface. Ciliated epithelial cells developed vacuolization of the apical and subnuclear cytoplasm and eventually fragmented along planes formed by coalescing vacuoles. Non-ciliated cells showed apical swelling and loss of microvilli during the course of infection. After degeneration and sloughing of both ciliated and non-ciliated cells, a flattened layer of intact basal cells covered the submucosa. It is likely that progressive vacuolization of epithelial cells leads to exfoliation of both cells and cell fragments in *M. pneumoniae* infection. Since organisms frequently are associated with these exfoliated cells, their potential presence in sputum and lavage specimens could prove to be of diagnostic importance.

Key words: *Mycoplasma pneumoniae* – Tracheal epithelium – Organ culture – Transmission and scanning electron microscopy.

Introduction

Mycoplasma pneumoniae is an important respiratory pathogen of man (Chanock 1965; Denny et al. 1971). It is the most common cause of pneumonia in children and young adults (Collier 1972). Since infection rarely is fatal, histopathological observations on the naturally occurring disease are lacking (Clyde 1973).

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Experimental *M. pneumoniae* infection of golden Syrian hamsters appears to simulate the disease in man (Dejani et al. 1965; Fernald 1969; Denny et al. 1971; Clyde 1973; Collier and Clyde 1974; Hara et al. 1974). Transmission electron microscopy (TEM) of organ cultures of hamster trachea has proven useful in assessing the interaction of *M. pneumoniae* with the ciliated respiratory epithelium (Collier et al. 1969, 1971; Collier and Baseman 1973; Woodruff 1973; Powell et al. 1976; Wilson and Collier 1976). However, knowledge of cellular alterations in the respiratory mucosa is incomplete.

Exfoliation of epithelial cells is observed after a variety of insults to the tracheobronchial mucosa (Harris et al. 1971; Dahlgren and Dalen 1972; Reed and Boyde 1972; Boatman et al. 1974; Muse et al. 1976; Mossman et al. 1977, 1978); the presence of these cells in sputum and lavage specimens can be of diagnostic importance (Papanicolaou 1956). Exfoliation of intact and fragmented cells has been observed in *M. pneumoniae* infection (Collier et al. 1969; Collier and Clyde 1971; Collier 1972; Muse 1976) although the mechanism and significance of this phenomenon are poorly understood.

In this study, TEM and scanning electron microscopy (SEM) were employed to examine the process whereby epithelial cells are exfoliated from *M. pneumoniae*-infected tracheal mucosa.

Materials and Methods

A recent clinical isolate from a patient with serologically established pneumonia due to *M. pneumoniae* was provided by Dr. Dieter Gump. Virulence was established by demonstrating its ability to produce pneumonia in hamsters after intranasal inoculation (Lippman et al. 1969; Collier and Baseman 1973). Colonies of organisms cultured in broth medium (Hayflick 1965) exhibited *M. pneumoniae* antibody-specific immunofluorescence (Collier and Clyde 1974).

Tracheal organ cultures were prepared from 3-week-old adult golden Syrian 15.16 strain hamsters (TELACO, Bar Harbor, ME) as described previously (Mossman and Craighead 1975). Respiratory tissues of animals from this source have failed to yield endogenous mycoplasma in our laboratory when appropriately tested. Culture medium consisted of Eagle's minimal essential medium (GIBCO, Grand Island, NY), 5% chicken serum (Microbiological Associates, Walkersville, MD), 5 mcg/ml Fungizone (GIBCO), and 100 units/ml of penicillin G (Lilly). The second broth passage of the isolate in the log phase of growth was used to infect cultures; the inoculum consisted of 0.25 ml of broth (Collier et al. 1969) containing approximately 5×10^7 colony-forming units. An equal amount of sterile broth was added to control cultures. All cultures were incubated at 36° C in a 95% air −5% carbon dioxide, water-saturated environment.

Explants from two control and two infected cultures were rinsed in parallel with Hanks' balanced salt solution (Reed and Boyde 1972) at 6-h intervals during the first day, and at 24-h intervals thereafter, for a total of 4 days. The tissues were submerged in Hayflick's medium briefly (to establish the presence of *M. pneumoniae* by culture) and then fixed in 4% buffered glutaraldehyde.

Explants from four identical, individually performed experiments were prepared for ultrastructural study. For TEM, glutaraldehyde-fixed tracheal hemisections were washed in 0.1 M cacodylate buffer, post-fixed in 2% osmium tetroxide, and embedded in Epon. Thick and thin sections were prepared for light microscopic (LM) and TEM study. Specimens were examined with a Philips 201 transmission electron microscope. For SEM, glutaraldehyde-fixed tissue was washed in 0.1 M cacodylate buffer, dehydrated in a graded series of ethanol solutions, and transferred through a gradient of Freon in ethanol. Tissue was critical point dried with a Bomar SPC apparatus and attached to specimen stubs with silver conductive paint. Specimens were examined with a JEOL JSM-35 scanning electron microscope.

Results

M. pneumoniae-inoculated tracheal organ cultures consistently yielded the organism at the time of harvest; control cultures were sterile in Hayflick's broth medium and exhibited normal cellular morphology by LM, TEM and SEM (Figs. 1a, 1b). Filamentous forms resembling *M. pneumoniae* by TEM and SEM appeared at the bases of cilia and adjacent to interepithelial junctions (Collier and Baseman, 1973; Muse et al., 1976) in increasing numbers (Table 1) during the first 48 h after inoculation (Figs. 2a, 2b). The organisms were approximately 250–400 nm in length. By TEM they were larger and more electron-dense than the microvilli of nearby non-ciliated epithelial cells.

Focal cytopathic alterations were found in the mucosa of infected tracheal explants 18 h after inoculation. These changes became more widespread with the passage of time (Table 1). By 48 h prominent supra- and subnuclear vacuolization distorted the cytoplasm of the ciliated cells, partially separating the apices from subjacent components (Figs. 3a, 3b). At this time, the nuclei exhibited margination of the chromatin and discrete nucleoli were not evident. In addition, the mitochondria appeared enlarged and the cristae disrupted. These changes became increasingly evident during the course of infection. By 72 h after inoculation, many cells with roughened apical surfaces could be seen by SEM, suggesting remnants of the bases of previously fragmented epithelial cells (Fig. 4a). Exfoliated apical fragments of ciliated cells were found concurrently (Fig. 4b).

Ciliated epithelial cells were more severely involved than non-ciliated cells at all stages of the infection. By 48 h after inoculation, the latter cells appeared enlarged and swollen; near the apices there was loss of microvilli (Figs. 3a, 5c). Many of these cells acquired a ruffled plasma membrane and protruded from the lumenal surface of the organ culture; others extruded mucinous material (Fig. 5c). Most were exfoliated by 72 to 96 h after inoculation.

Basal cells failed to exhibit the alterations observed in the superficial ciliated and non-ciliated cells. In the later stages of infection (72–96 h), TEM and SEM revealed necrotic, exfoliated epithelial cells and cell fragments resting upon a continuous monolayer of flattened basal cells that appeared to cover the subjacent basement membrane and mucosa (Fig. 6 and inset).

Discussion

This study elucidates the mechanism of epithelial cell injury in *M. pneumoniae* infection in vitro and corroborates previous TEM (Collier et al. 1969, 1971, 1973) and SEM (Muse et al. 1976) investigations. By examining tissue by both TEM and SEM at sequential stages in the course of the infection, it was possible to correlate surface changes with intracellular events. In addition, SEM permitted the examination of relatively large areas of epithelium, obviating the sampling problems encountered by the use of TEM alone.

The characteristic filamentous morphology of *M. pneumoniae* readily is identifiable by TEM (Collier et al. 1971; Collier and Baseman 1973; Woodruff 1973; Powell 1976; Wilson and Collier 1976) and SEM (Biberfeld and Biberfeld

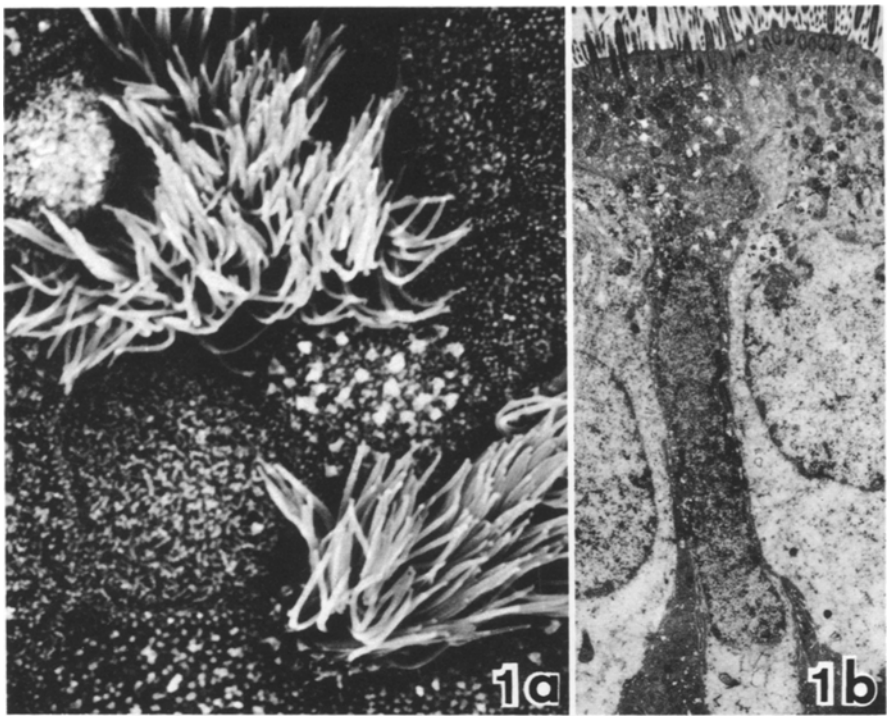

Fig. 1. **a** Scanning electron micrographs (SEM) of uninfected trachea after 96 h in organ culture. Cell surfaces are covered with cilia and microvilli and intercellular junctions are intact. (× 3,500). **b** Transmission electron micrograph (TEM) of uninfected trachea after 96 h in organ culture. Small cytoplasmic lucencies and basal swelling are apparent. (× 5,000)

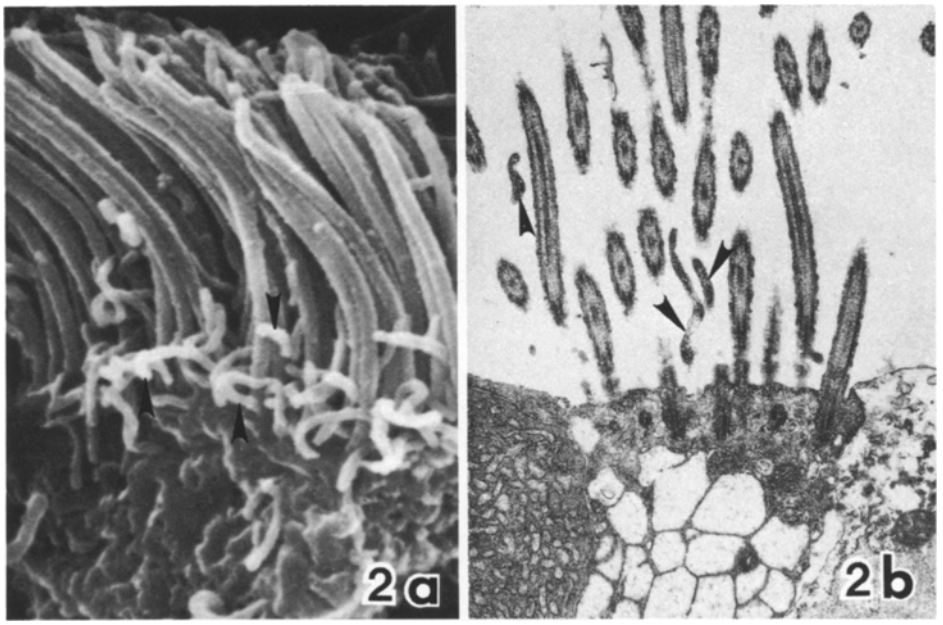

Fig. 2. **a** SEM of ciliated epithelial cell 24 h after inoculation with *M. pneumoniae*. Filamentous organisms are concentrated at ciliary bases (*arrowheads*). (× 12,000). **b** TEM of ciliated epithelial cell 24 h after inoculation. Note electron-dense organisms (*arrowheads*) at base of cilia and prominent apical vacuolization. (× 26,000)

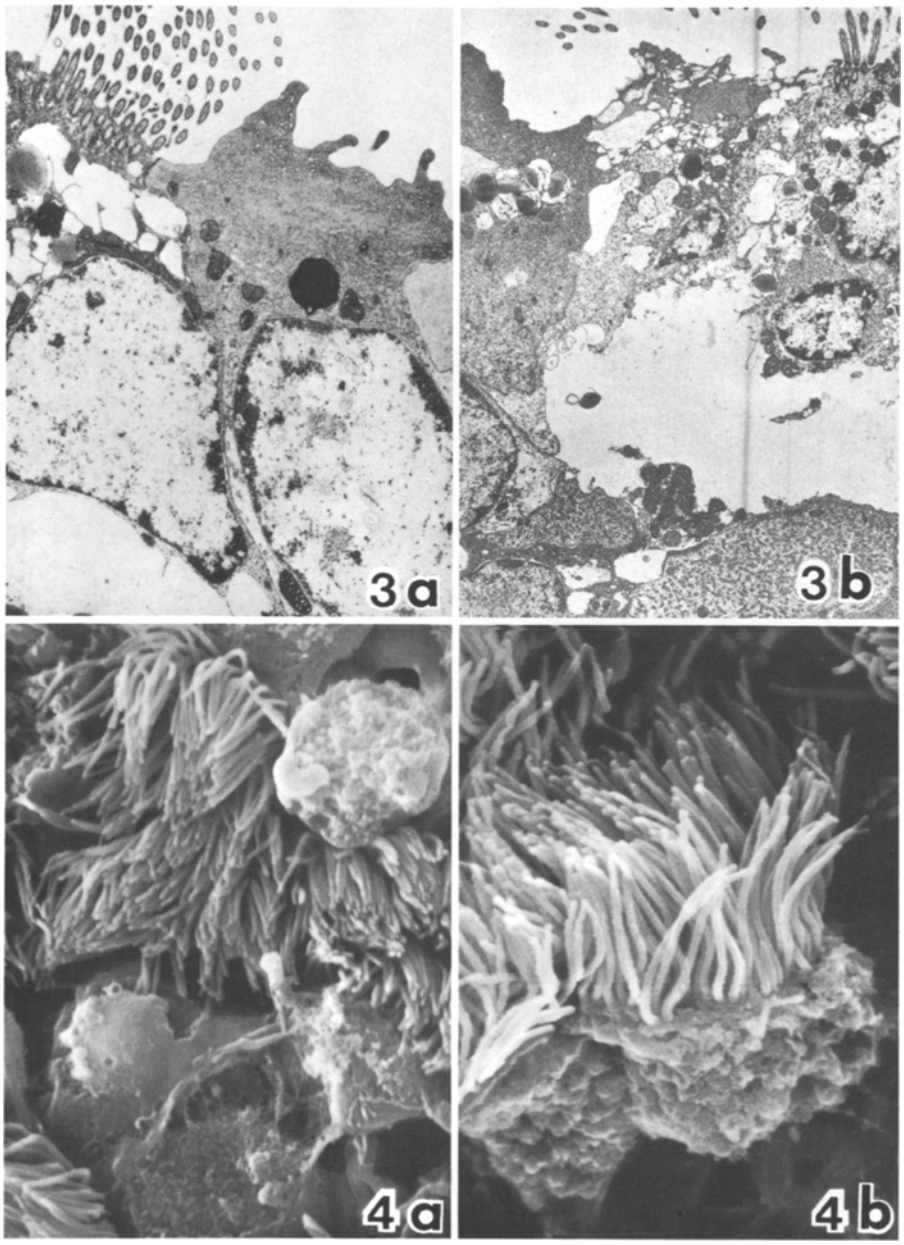

Fig. 3. a TEM of tracheal epithelium 48 h after inoculation shows infected ciliated cell with enlarged nucleus and mitochondria and extensive apical and subnuclear vacuolization of the cytoplasm. An adjacent non-ciliated cell is relatively intact and protrudes into the lumen. (×6,000). **b** TEM of tracheal epithelium 48 h after inoculation. Note the fragmentation of ciliated cells and the dissociation from intact basal cells. (×4,000)

Fig. 4. a SEM 48 h after inoculation shows roughened surfaces that correspond to the fragmented portions of the ciliated epithelial cells. (×3,500). **b** Free apical fragments of ciliated cells 48 h after inoculation. (×6,000)

Table 1. Semiquantitative characterization of progressive alteration in ciliated cells of hamster tracheal organ cultures after inoculation with *M. pneumoniae*

Interval after inoculation (h)	Percentage of ciliated cells with associated organisms	Pathologic alterations		
		Mucosal involvement (% surface area)	Nuclei[a]	Cytoplasmic vacuolization[b]
6	10	0	–	–
12	10–25	0	–	–
18	50	25	–	slight
24	75	75–100	slight	severe
48	100	100	moderate to severe	severe, with cell fragmentation
72	100	100	severe	severe, with exfoliation
96	100	100	severe	superficial cells totally absent

[a] Defined as nuclear enlargement (slight); nuclear enlargement with peripheral margination of chromatin and inconspicuous or absent nuclei (moderate); or as the aforementioned alterations in association with disruption of the nuclear membrane (severe)

[b] Defined as multiple small, discrete cytoplasmic vacuoles (slight); multiple, focally coalescent vacuoles (moderate); or multiple coalescent vacuoles forming planes of cell fragmentation (severe)

1970; Muse 1976). Our observations on the association of these organisms with ciliated epithelial cells are consistent with the findings recorded in previous reports (Collier et al. 1969, 1971; Collier 1972; Collier and Baseman 1973; Muse et al. 1976; Powell et al. 1976). In this study, numerous organisms were located adjacent to, and on the surfaces of, ciliated cells that exhibited lesions. The organisms were not intimately associated with the non-ciliated cells, even though they exhibited cytopathic changes.

Fragmentation and exfoliation of ciliated epithelial cells has been described previously in *M. pneumoniae* infection (Collier et al. 1969; Collier and Clyde 1971; Collier 1972; Muse et al. 1976), *Bordetella pertussis* infection (Muse et al. 1977), viral infections (Reed and Boyde 1972), and after exposure to ozone (Boatman et al. 1974), ferric oxide (Harris et al. 1971), acrolein (Dahlgren and Dalen 1972), sulfur dioxide (Asmundsson et al. 1973) and inorganic particulates (Mossman et al. 1977, 1978). In the infected cultures we examined, coalescence of vacuoles in the cytoplasm of ciliated epithelial cells resulted in fragmentation and sloughing. It has been suggested that the exfoliation of these cells resembles the process of ciliocytophthoria (Muse 1976), first described by Papanicolaou (1956). Our study corroborates this observation. Since similar cytopathic changes have been described previously after exposure to chemical and particulate respiratory irritants (Dahlgren and Dalen 1972; Asmundsson et al. 1973; Basrur and Basrur 1976; Mossman et al. 1977), ciliocytophthoria appears to represent a non-specific response to a variety of environmental insults.

Alterations in mitochondria similar to those cited herein also have been noted in organ cultures of human fetal trachea infected with *M. pneumoniae*

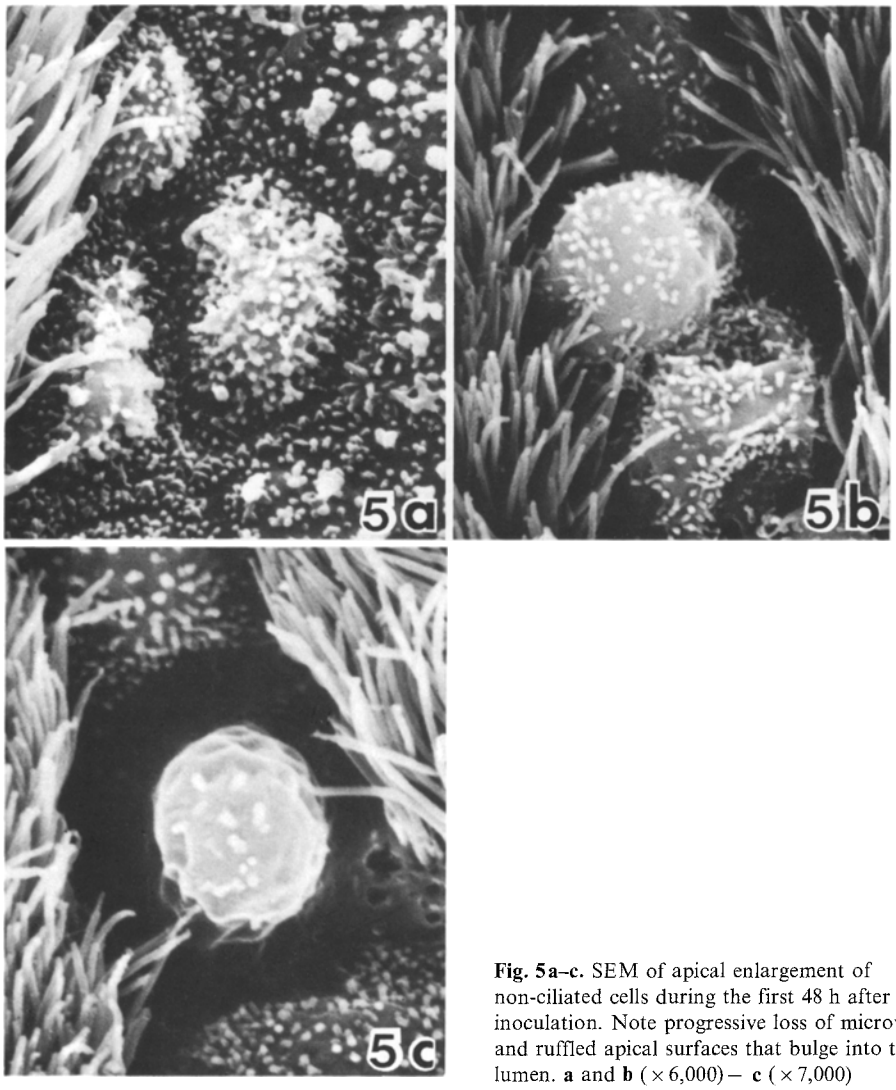

Fig. 5a–c. SEM of apical enlargement of non-ciliated cells during the first 48 h after inoculation. Note progressive loss of microvilli and ruffled apical surfaces that bulge into the lumen. **a** and **b** (×6,000) – **c** (×7,000)

(Collier and Clyde 1971; Collier 1972). Since infection with *M. pneumoniae* leads to ciliostasis (Collier et al. 1969, 1971; Collier and Clyde 1971; Collier 1972), the mitochondrial changes may reflect a biochemical basis for impaired ciliary function. Hu (1975) has shown that the hamster trachea infected with *M. pneumoniae* exhibits an inhibition of host cell RNA and protein synthesis by 24 h after inoculation. The nuclear and mitochondrial changes described in the present study temporally correlate with these biochemical events.

The cytolytic changes demonstrated in the differentiated surface epithelial cells were not present in basal cells. In this and previous studies, basal cells of the trachea were found to assume a flattened, squamous appearance late

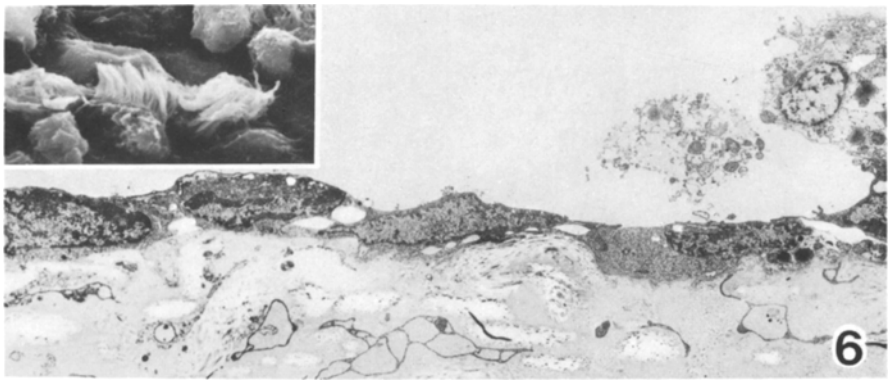

Fig. 6. TEM and SEM (insert) of tracheal mucosa 96 h after inoculation. Note the attenuated single layer of basal cells covering the submucosa. Necrotic fragments of exfoliated epithelial cells are present above this basal layer. TEM ($\times 3{,}000$) – SEM ($\times 1{,}000$)

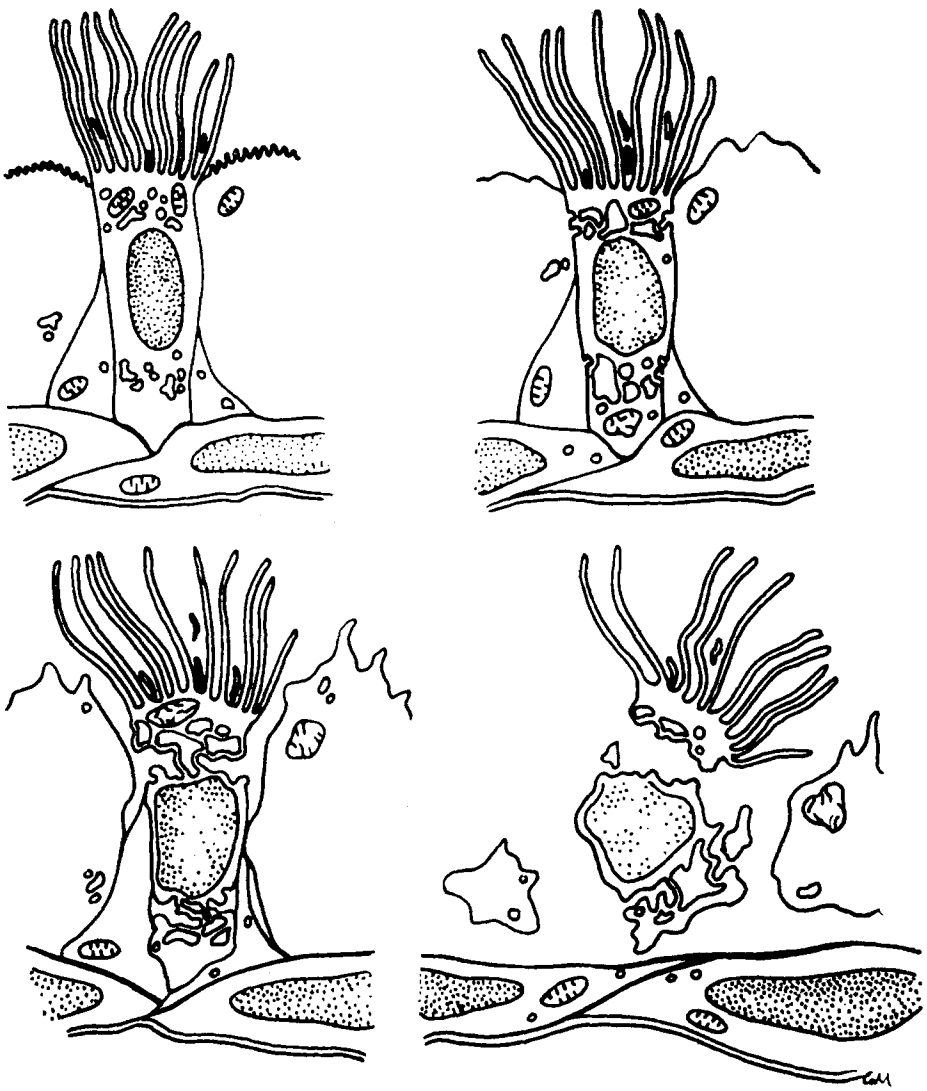

Fig. 7. Representation from ultrastructural data of sequential alterations in ciliated epithelial cells. Formation of coalescing cytoplasmic vacuoles eventuates in fragmentation and exfoliation of ciliated cells. Intact basal cells are not affected and cover the submucosa

in the course of *M. pneumoniae* infection (Clyde 1973; Hara et al. 1974). Respiratory basal cells have been shown to be resistant to a wide variety of insults, including viruses (Craighead 1968), mineral particulates (Mossman et al. 1977), and polycyclic hydrocarbons (Mossman and Craighead 1974). The regenerative potential of basal cells in *M. pneumoniae* infection has not been defined and warrants further investigation.

Although cytopathic alterations in upper airway mucosa could potentially contribute to symptomatic tracheobronchitis in naturally infected humans, compelling experimental and seroepidemiological evidence implicates immune mechanisms in the pathogenesis of *M. pneumoniae* disease (Smith et al. 1967; Fernald 1972, 1973; Fernald and Glezen 1973; Craighead 1975). Thus, direct cytopathic changes in the epithelium may contribute only in part to the clinical disease. Since organisms often are associated with exfoliated ciliated epithelial cells, further investigation is needed to define the potential diagnostic value of exfoliated cells in sputum and lavage specimens from patients with suspected *M. pneumoniae* infection.

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References

Asmundsson T, Kilburn KH, McKenzie WN (1973) Injury and metaplasia of airway cells due to SO_2. Lab Invest 29:41–53

Basrur PK, Basrur VR (1976) Respiratory surface epithelium: Scanning electron microscopic technique for bioassay on inhalants. In: Scanning Electron Microscopy. IITRI 1976, Chicago, p 623

Biberfeld G, Biberfeld P (1970) Ultrastructural features of *M. pneumoniae*. J Bacteriol 102:855–861

Boatman ES, Sato S, Frank R (1974) Acute effects of ozone on cat lungs. Am Rev Respir Dis 110:157–169

Chanock RM (1965) Mycoplasma infections in man. N Engl J Med 273:1199–1206

Clyde WA (1973) Models of *M. pneumoniae* infection. J Infect Dis 127:S69–72

Collier AM (1972) Pathogenesis of *M. pneumoniae* infection as studied by human foetal trachea in organ culture. In: Pathogenic Mycoplasmas, Assoc Scientific Pub, Amsterdam, p 307

Collier AM, Baseman JB (1973) Organ culture techniques with mycoplasmas. Ann NY Acad Sci 225:277–289

Collier AM, Clyde WA (1971) Relationship between *M. pneumoniae* and human respiratory epithelium. Infec Immun 3:694–701

Collier AM, Clyde WA (1974) Appearance of *M. pneumoniae* in lungs of experimentally infected hamsters and sputa from patients with natural disease. Am Rev Respir Dis 110:765–773

Collier AM, Clyde WA, Denny FW (1969) Biologic effects of *M. pneumoniae* and other mycoplasmas from man on hamster tracheal organ culture. Proc Soc Exp Biol Med 132:1153–1158

Collier AM, Clyde WA, Denny FW (1971) *M. pneumoniae* in hamster tracheal organ culture: Immunofluorescent and electron microscopic studies. Proc Soc Exp Biol Med 136:569–573

Craighead JE (1975) Report of a workshop: Disease accentuation after immunization with inactivated microbial vaccines. J Infect Dis 131:749–753

Craighead JE (1968) Cytopathic effects of parainfluenza type 3 in organ cultures of human respiratory tract tissue. Am J Pathol 52:287–300

Dahlgren SE, Dalen H (1972) Ultrastructural observations of chemically induced inflammation in guinea pig trachea. Virchows Archiv [Cell Pathol] 11:211–223

Dejani AS, Clyde WA, Denny FW (1965) Experimental infection with *M. pneumoniae* (Eaton's agent). J Exp Med 121:1071–1086

Denny FW, Clyde WA, Glezen WP (1971) *Mycoplasma pneumoniae* disease: Clinical spectrum, pathophysiology, epidemiology and control. J Infect Dis 123:74–92

Fernald GW (1969) Aspects of experimental *M. pneumoniae* infection. J Infect Dis 119:255–266

Fernald GW (1972) In vitro response of human lymphocytes to *Mycoplasma pneumoniae*. Infec Immun 5:552–558

Fernald GW (1973) Role of host response in *M. pneumoniae* disease. J Infect Dis 127:55–58

Fernald GW, Glezen WP (1973) Humoral and cellular immune responses to an inactivated *Mycoplasma pneumoniae* vaccine in children. J Infect Dis 127:498–504

Hara K, Izumikawa K, Kinoshita I, Ota M, Ikebe A, Koike M, Hamada M (1974) Experimental infection with *M. pneumoniae* in young hamster: Location of ferritin-labeled antibody binding to infective tissue. Tohoku J Exp Med 114:315–337

Harris CC, Sporn MG, Kaufman DG, Smith JM, Baker MS, Saffiotti U (1971) Acute ultrastructural effects of benzo (a) pyrene and ferric oxide on the hamster tracheobronchial epithelium. Cancer Res 31:1977–1989

Hayflick L (1965) Tissue cultures and mycoplasmas. Tex Rep Biol Med 23:S285–303

Hu PC, Collier AM, Baseman JB (1975) Alterations in the metabolism of hamster tracheas in organ culture after infection with virulent *M. pneumoniae*. Infec Immun 11:704–710

Lippman RP, Clyde WA, Denny FW (1969) Characteristics of virulent, attenuated and avirulent *M. pneumoniae* strains. J Bacteriol 100:1037–1043

Mossman BT, Adler KB, Craighead JE (1978) Interaction of carbon particulates with tracheal epithelium in organ culture. Environ Res 16:110–122

Mossman BT, Craighead JE (1974) Topical application of polycyclic hydrocarbons to differentiated respiratory epithelium in organ culture. In: Experimental Lung Cancer. Carcinogenesis and Bio-Assays, Springer, Berlin, p 514

Mossman BT, Craighead JE (1975) Long-term maintenance of differentiated respiratory epithelium in organ cultures. I. Medium composition. Proc Soc Exp Biol Med 149:227–233

Mossman BT, Kessler JB, Ley BW, Craighead JE (1977) Interaction of crocidolite asbestos with hamster respiratory mucosa in organ culture. Lab Invest 36:131–139

Muse, KE, Collier AM, Baseman JB (1977) Scanning electron microscopic study of hamster tracheal organ cultures infected with *Bordetella pertussis*. J Infect Dis 136:768–777

Muse KE, Dwight AP, Collier AM (1976) *M. pneumoniae* in hamster tracheal organ culture studied by scanning electron microscopy. Infec Immun 13:229–237

Papanicolau GN (1956) Degenerative changes in ciliated cells exfoliating from bronchial epithelium as a cytologic criterion in diagnosis of diseases of the lung. NY State J Med 56:2647–2650

Powell DA, Hu PC, Wilson M, Collier AM, Baseman JB (1976) Attachment of *M. pneumoniae* to respiratory epithelium. Infect Immun 13:959–966

Reed SE, Boyde A (1972) Organ cultures of respiratory epithelium infected with rhinovirus or parainfluenza virus studied by a scanning electron microscope. Infec Immun 6:68–76

Smith CB, Friedewald WT, Chanock RM (1967) Inactivated *Mycoplasma pneumoniae* vaccine: evaluation in volunteers. JAMA 199:353–358

Wilson MH, Collier AM (1976) Ultrastructural study of *Mycoplasma pneumoniae* in organ culture. J Bacteriol 125:332–339

Woodruff KH (1973) Ultrastructural changes in hamster tracheal ring cultures exposed to *M. pneumoniae*. Am J Pathol 72:91–102

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