Relationships Between *Mycoplasma pneumoniae* and Human Respiratory Epithelium

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The interaction was studied between *Mycoplasma pneumoniae* and its natural host cell, the human respiratory epithelium. Organized, ciliated cells provided by fetal trachea in organ culture enabled examination of the host-parasite relationship by light, immunofluorescence, and electron microscopy. Impairment of cellular function was reflected by disorganization and loss of ciliary motion; this was associated with a sequence of cytopathological changes denoting progressive cell injury. The organisms were found concentrated on the luminal surface of ciliated epithelium and cells lining the submucosal glands. A differentiated portion of the *Mycoplasma*, consisting of an extension of the unit membrane containing an electron-dense core surrounded by a lucent space, served as the means of attachment to host cells. The findings suggest that the pathogenicity of *M. pneumoniae* depends upon intimate extracellular infection with production of functional and structural changes initiated by host cell membrane injury.

The nature of the interaction between *Mycoplasma pneumoniae* and host cells is incompletely understood, limiting insight into the pathogenesis of the common respiratory illnesses caused by this organism. The very low mortality of infections in man has provided little pathological material for study; this has necessitated the use of a variety of experimental models, none of which is completely satisfactory, for analysis of the disease mechanisms. Previous reports from this laboratory have demonstrated that tracheal organ culture provides a model system in which organized ciliated respiratory epithelium can be maintained, infected with mycoplasmas, and observed in a controlled environment (5, 6). The studies to be described concern the relationship between *M. pneumoniae* and its natural target cell, the human ciliated respiratory epithelium. These specialized host cells were provided by establishing fetal trachea in organ culture.

MATERIALS AND METHODS

Medium. Tissue culture medium 199 with 20% fresh human serum, 10% yeast extract, 1% dextrose, 0.005% phenol red, 1.2% *N*-2-hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid (HEPES) buffer (Calbiochem, Los Angeles, Calif.), and 1,000 units of penicillin G per ml was used to support the organ cultures and allow growth of the organisms.

Organisms. The *M. pneumoniae* strain used was recovered from a patient with pneumonia (M129) and passed 10 times in Hayflick's broth medium (9).

The organism was virulent as defined by its ability to produce pneumonia in the Syrian hamster (12). For limited comparative studies, other human *Mycoplasma* species were employed: *M. fermentans* (PG-18), *M. salivarium* (PG-20), *M. hominis* (DC-63), and *M. orale* type 1 (Patt strain, formerly designated *M. pharyngis*). These organisms were propagated and quantitated as previously described (11).

Organ cultures. Tracheas from human fetuses of 16 to 20 weeks gestation were used in these studies. To insure sterility of tissues, specimens were chosen only from hysterotomies performed because of psychiatric indications. The trachea from the level of just below the larynx to just above the carina was exposed and removed under aseptic conditions. Transverse sections containing one cartilage ring were prepared and placed in plastic petri dishes (no. 1006, Falcon Plastics, Los Angeles, Calif.) with 2.25 ml of medium, incubated at 36 C in air with 5% CO₂, and observed as previously described (5). The organ cultures were inoculated with 0.25 ml of log-phase *Mycoplasma* cultures or with sterile medium as a control.

Histopathology. The methods used for examination of infected tracheal organ cultures have been described in detail elsewhere (5, 6). Comparable specimens were fixed in Van de Grift's solution for light microscopy, quick-frozen in O.C.T. compound (Ames Co., Elkhart Ind.) for immunofluorescence, or treated with glutaraldehyde in phosphate buffer followed by OsO₄ in Veronal-acetate buffer for electron microscopy.

RESULTS

Ciliary activity in Mycoplasma-infected tracheal epithelium. Human tracheal organ cultures re-

mained viable, as indicated by continuation of organized ciliary activity, as long as 4 weeks when the medium was changed every 4 days. To examine the effects of *Mycoplasma* infection, experiments were performed in which a series of separate organ cultures prepared with sections from the same trachea were inoculated with several human *Mycoplasma* species. Ciliary motion was observed daily, and organisms were quantitated at the beginning and at the end of the observation period (Table 1). Ciliary activity in the cultures inoculated with *M. pneumoniae* slowed after 3 days and was absent on day 4. No apparent effect was seen in the organ cultures inoculated with *M. hominis*, *M. salivarium*, *M. orale* (type 1), *M. fermentans*, or in sterile control cultures. In other experiments, no detectable changes resulted from infection of tracheal sections with an avirulent *M. pneumoniae* strain (12), inoculation with heat-killed organisms, or addition of sterile culture filtrates to the system. These findings provide evidence that virulent *M. pneumoniae* is unique among the mycoplasmas tested in its ability to interfere with an important function of human respiratory epithelium.

Light microscopic examination of tissues. The preceding experiments were indicative of tissue injury by *M. pneumoniae* as revealed by physiological disturbances accompanying infection. The nature of this injury was studied by removing specimens from the culture system at intervals for histological examination. A section of uninoculated human trachea, maintained for 48 hr in vitro, is shown in Fig. 1A. Features of the tissue which could be identified were the pseudostratified ciliated columnar epithelium, the lamina propria with numerous submucosal glands, cartilage, and serosa. The earliest changes, occurring 48 hr after inoculation with *M. pneumoniae* (Fig. 1B), included epithelial cell cytoplasmic eosinophilia and vacuolization, nuclear swelling, and chromatin margination. These changes were also present in the epithelial cells lining the submucosal glands. Eosinophilic material, which was not seen in the control specimens, was noted at the base of the cilia; a palisading effect appeared to occur between the cilia and these eosinophilic deposits (Fig. 1C). At 72 hr, with further progression of infection, complete loss of cilia was apparent in many cells (Fig. 1D). Other cells protruded into the lumen, with distortion of the cilia into a sunburst pattern. At 96 hr, the entire epithelial layer became disorganized with loss of polarity of individual cells (Fig. 1E). At times, the remaining epithelium took on a squamous appearance and masses of eosinophilic material occurred on the luminal surface (Fig. 1F). These findings indicated progressive cell injury and suggested that

TABLE 1. Effect of five human *Mycoplasma* species on the ciliary activity of human fetal trachea in organ culture

Species	CFU/ml (log ₁₀) ^a		Ciliary activity ^b				
	Day 0	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
<i>M. pneumoniae</i> ...	5.40	7.15	+	+	±	—	—
<i>M. hominis</i>	5.88	7.28	+	+	+	+	+
<i>M. salivarium</i> ...	6.88	6.81	+	+	+	+	+
<i>M. orale</i> type 1..	5.40	6.38	+	+	+	+	+
<i>M. fermentans</i> ...	6.18	6.38	+	+	+	+	+
Control.....			+	+	+	+	+

^a Colony-forming units per milliliter of culture fluid.

^b Present (+) or absent (—).

microcolonies of the *Mycoplasma* developed in intimate association with the epithelial cells. The occurrence of cytopathology in this system confirms a preliminary observation by Butler; however, no details were provided and it is not clear that the changes seen were the consequence of infection (2).

Immunofluorescent studies. To differentiate organisms from cellular debris or precipitated protein and to localize the antigen in relation to the damaged cells, indirect fluorescent-antibody staining was carried out by using *M. pneumoniae* rabbit antisera and goat anti-rabbit globulin conjugated with fluorescein isothiocyanate. Tissue at 48 hr after inoculation showed bright, specific fluorescence limited mainly to the ciliated border (Fig. 2A). The palisade appearance between the unstained cilia and the antigen paralleled the changes observed with the light microscope (Fig. 1C). Tissue at 72 hr after inoculation showed an increase in the amount of fluorescence at the luminal border (Fig. 2B). Fluorescence was also noted to extend down into the submucosal glands, where cytopathological changes had been demonstrated (Fig. 1B). No specific fluorescence was observed in control preparations which included infected tissue sections reacted with the fluorescein-conjugated antiglobulin (alone or preceded by normal rabbit serum) and normal tissue treated with antiserum and labeled antiglobulin.

The exact relationship between individual organisms and the ciliated cells could not be resolved by these immunofluorescence methods. Since the majority of the epithelial cells appeared to be parasitized, it was felt that electron microscopy would be feasible to provide further information.

Ultrastructural studies. The ultrastructure of adult human tracheal epithelium has been studied by Rhodin (15). However, there have been no

FIG. 1. Human fetal trachea infected in organ culture with *Mycoplasma pneumoniae* (hematoxylin and eosin stain). (A) Control trachea maintained in organ culture for 48 hr. (B) Cytoplasmic vacuolization of luminal and submucosal gland (sg) epithelium, 48 hr postinfection. Bm, basement membrane. (C) Deposits of eosinophilic material at the base of the cilia, 48 hr postinfection. Cell nuclei are enlarged with marginated chromatin. (D) Cilia are lost from many cells or distorted into a sunburst pattern, 72 hr postinfection. (E) Disorganization of epithelial layer, 96 hr postinfection. (F) Granular, eosinophilic microcolony on the surface of remaining undifferentiated epithelium, 96 hr postinfection. Bars in B to F, 10 μ m.

FIG. 2. Human fetal trachea infected in organ culture with *Mycoplasma pneumoniae* and reacted with *M. pneumoniae* antiserum (indirect immunofluorescent technique). (A) Specific fluorescence limited to ciliated epithelium (ce) 48 hr postinfection. Bm, basement membrane. (B) Increased fluorescence at the ciliated epithelial border and extending into the submucosal gland (sg), 72 hr postinfection. Bar, 20 μ m.

reports of detailed studies of human fetal trachea or of changes that may occur during organ culture. Examination of tissue maintained in organ culture for as long as 48 hr (Fig. 3A) revealed that detailed ultrastructure was present which resembled findings reported for the adult trachea. The fibrillar structure of the cilia, numerous branching microvilli, and the endoplasmic reticulum were well preserved (Fig. 3B). Forty-eight hours after inoculation of organ cultures (Fig. 3C), numerous filamentous organisms were seen among the cilia, in correspondence to the distribution of *M. pneumoniae*-specific antigen demonstrated by immunofluorescence. In the thin sections examined, as many as 11 organisms could be found at the base of the cilia across the width of a single epithelial cell. More distally the inter-ciliary spaces were packed with organisms cut in various planes. This may account for the sunburst configuration of cilia noted by light microscopy (Fig. 1D). No organisms were found within cells in any specimens examined, nor were they found in uninoculated control tissues.

The organisms were seen to have a distinctive morphological feature, consisting of a differentiated terminal structure. This portion of the *Mycoplasma* was always found in close proximity to the host cell membrane; five examples are demonstrated in the 2.2 μ m² field represented by Fig. 3D. The regular orientation of this structural feature toward the cell surface suggests the mode of organism attachment. The frequency with

which this finding was observed indicated that it was not based on random distribution of organisms with respect to the tissue.

A sagittal section of a complete organism is shown in Fig. 4A. The organism depicted measures 0.075 by 1.8 μ m, the tapered terminal tip accounting for about 10% of the length of the organism. The specialized ending was also seen in organisms used as the inoculum for these experiments, as demonstrated in Fig. 4B and C. Thin sections were prepared of pellets fixed after centrifugation of log-phase culture at 15,000 $\times g$ for 30 min. In this case, the terminal structures were noted infrequently, probably indicating random orientation of organisms to the plane of section rather than lack of differentiation. The filamentous morphology of the organism was less evident in the pellets as well.

The relationship between the host cell membrane and the organism is illustrated further in Fig. 5. In Fig. 5A, the appearance of three organisms cut in slightly different planes suggests that the tip structure consists of an electron-dense core surrounded by a lucent area. A cross section of the structure is indicated by the arrow in Fig. 5B. Very close approximation of host cell and organism membrane was noted only at the side or end of the specialized tip, providing further evidence of the importance of this device for attachment to host cells. In some instances, the epithelial membrane appeared indistinct at the point of organism contact, suggesting the possibility of local

FIG. 3. Electron photomicrographs of human fetal trachea. (A and B) Uninfected tissue maintained in organ culture for 48 hr: is, intercellular space; mv, microvilli; ci, cilia. (C and D) Tracheal tissue infected in organ culture with *Mycoplasma pneumoniae* for 48 hr. *Mycoplasma* cells (my) appear as pleomorphic electron-dense structures at the cell surface and among the cilia. Five examples of the differentiated portion of the organism (arrows) are shown in D. Bars, 1 μ m.

injury (Fig. 5A and D). At greater magnification (Fig. 6), the triple-layered unit membrane of the organism could be seen to encompass the dense core and lucent space of the attached segment. In addition, the central core appeared to have a filamentous structure.

DISCUSSION

Specificity of host cells for infection by *M. pneumoniae* is implied by the occurrence of

natural disease only in man. The common clinical manifestations of disease—including symptoms and signs of tracheitis or tracheobronchitis—imply further that the ciliated respiratory epithelium is the chief cell type which is parasitized. In the present studies, an organ culture system was used to maintain viable human ciliated tracheal epithelium in which the effects of *M. pneumoniae* infection could be observed at the cellular level. Use of this isolated system provided an indication

of the direct pathogenicity of the organism by revealing (i) interference with ciliary motility and synchrony, (ii) a sequential pattern of cytological changes, and (iii) disruption of normal tissue architecture. Since natural *M. pneumoniae* disease is a mild but protracted illness, the destructive effects of the organism for tracheal cells are probably modified by the normal defenses of the intact host.

Adsorption of *M. pneumoniae* to a variety of other human cells has been demonstrated, including cervical epithelium (HeLa cells), spermatozoa, erythrocytes, and leukocytes (17, 18; Del Giudice and Pavia, Bacteriol. Proc., p. 71, 1964). It is not clear that multiplication of organisms occurs in association with the cells in these examples. Target cell specificity appears incomplete, however, since experimental infection can be established in a limited number of animal hosts; in addition, adsorption between nonhuman tracheal cells and the organisms has been reported (16). The host cells involved may share the sialic acid-like sites which are utilized by the organisms for

attachment. This adsorption mechanism has not been demonstrated in other human *Mycoplasma* species (17), perhaps explaining the lack of pathogenicity of selected species for cultured human trachea which was observed.

Results of the present studies suggest that the attachment mechanism of *M. pneumoniae* resides in a specialized terminal structure which measures approximately 80 to 100 by 250 to 300 nm. This structure is enclosed by the unit membrane of the organism and has an organized internal structure. The electron density and fibrillar nature of the central core suggest that it may contain a concentration of nucleic acids. The lucent area surrounding the core could be composed of lipid materials, since these do not stain with the techniques employed here. Whether the specialized tip has functions other than attachment remains to be determined. It is known that important antigenic determinants of *M. pneumoniae* are lipids (10), and the replicative mode of the organism is thought to be by binary fission (8). Another *Mycoplasma* species with demonstrated asymmetry is *M. gallisepticum* (13); in this case, a terminal bleb appears associated with attachment to host cells (18), although organism division seems to occur at the opposite pole (14).

The finding of a special morphological feature in *M. pneumoniae* grown in broth differs from results of previous studies on the ultrastructure of this *Mycoplasma* but confirms the findings of Biberfeld and Biberfeld which were reported recently (1). These authors described in detail the differentiated terminal portion of the organism, although their studies conducted in vitro did not suggest the functional significance of this structure. Further study of the specialized tip can be facilitated by use of a system containing host cells. As demonstrated by the present studies, the orientation of the organism appears to be regulated by the presence of host cells, in contrast to random positioning in material centrifuged from cell-free medium. From the frequency with which attached organisms were seen in thin sections, it can be calculated that over 100 could be present on the luminal surface of a single epithelial cell and countless more on the ciliary portion of the cell membrane. Specimens of this type would be valuable for combined histochemical and electron microscopic study.

The exact means by which *M. pneumoniae* injures host cells is unknown. It has been suggested that the generation of peroxide in close proximity to the cell membrane is of primary importance (4), and this has been documented in the case of *M. mycoides* var. *capri* (3). However, it has been reported that *M. pneumoniae* strains which are

FIG. 4. Electron photomicrographs of *Mycoplasma pneumoniae*. (A) Two organisms sectioned longitudinally, showing filamentous morphology and differentiation of ends attached to an epithelial cell. (B and C). Examples of specialized terminal structure (arrows) in organisms from log-phase broth culture used to inoculate organ cultures. Bars, 100 nm.

FIG. 5. (A-D) Electron photomicrographs of human fetal trachea infected in organ culture with *Mycoplasma pneumoniae* for 48 hr. Examples of the close association between the specialized tip of the organism and the cell membrane, cilia (ci), and microvilli (mv) are demonstrated. A transverse section of the specialized tip is seen in B (arrow). Bar, 100 nm.

attenuated in terms of their ability to produce pneumonia in the hamster have no diminution in peroxide production (12). Additionally, the degree of host cell damage is influenced by the cell type. No cytopathological changes are seen in infected chick embryo bronchial epithelium (7); in hamster tracheal organ cultures, marked destructive effects have been described, including disruption of epithelial terminal bars and invasion of the intercellular spaces by the parasite (6). Direct effects of the organism on human tracheal cells appear to fall between these two extremes. Since the *Mycoplasma* produces only an extra-

cellular infection in these instances, it is likely that pathogenic effects are dependent upon host cell membrane injury. Whether the mediator of injury is peroxide, alone or in concert with other toxic factors, remains to be determined.

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FIG. 6. Electron photomicrograph of human fetal trachea infected in organ culture with *Mycoplasma pneumoniae* for 48 hr. The differentiated tip of two organisms is seen between a microvillous (mv) and a cilium (ci). The central dense core and lucent surrounding area of the tip are enveloped by an extension of the unit membrane of the organism. An intact terminal bar (tb) connecting the luminal surface of two epithelial cells is also demonstrated. Bar, 100 nm.

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