Biologic Effects of *Mycoplasma pneumoniae* and Other Mycoplasmas from Man on Hamster Tracheal Organ Culture[1] (34385)

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Several experimental models have been used to study the host–parasite relationship in *Mycoplasma pneumoniae* disease. A variety of tissue-culture systems support growth of the organism (1); however, these cells lack the specialized function and differentiation of the natural "target cell" of this pathogen, the ciliated respiratory epithelium. *Mycoplasma pneumoniae* has also been propagated in the respiratory tract of chick embryos and will produce penumonia in hamsters and cotton rats (2). While these *in vivo* models provide specialized host cells, they do not permit continual observation and the disease is influenced by defense mechanisms of the intact animal. Applicability of tracheal organ culture to studies of respiratory viral agents (3) suggested that a similar model could be useful in investigation of mycoplasma infections, thereby circumventing the problems cited. This report indicates the use of hamster trachea in organ culture for the study of *M. pneumoniae* and reveals the unique nature of changes produced by this organism in comparison to other human mycoplasma species which were examined.

Materials and Methods. Media. Hayflick's broth medium (4), composed of 70% Difco PPLO broth, 20% fresh horse serum, 10% yeast extract, 1% dextrose, 0.005% phenol red, and 1000 units of penicillin G/ml, was used to propagate the organisms and support the organ cultures.

Organisms. Mycoplasma strains used in these studies and their sources included: strains PG-18 *M. fermentans* and PG-20 *M. salivarium* (R. M. Chanock); Patt strain *M. pharyngis* (W. A. Clyde); DC-63 strain *M. hominis* (M. A. Mufson); M129 strain *M. pneumoniae* (W. A. Clyde); and Mac strain *M. pneumoniae* (C. Liu). The M129 strain of *M. pneumoniae* was classified as virulent, and the Mac strain as attenuated, in terms of their ability to produce pneumonia in the Syrian hamster (5–7).

Organ Cultures. Eighty- to 100-g Syrian hamsters obtained locally were anesthetized with 10 mg of sodium pentobarbital intraperitoneally. The trachea from the level of just below the larynx to just above the carina was exposed and removed under aseptic conditions. The excised trachea was placed in Hayflick's broth and transverse sections about 1 mm in thickness, each containing one cartilage ring, were prepared. Each trachea yielded 14 to 16 rings of tissue. Up to six tracheal rings were placed in 50 × 12-mm Falcon plastic petri dishes on small areas of crosshatch scratches which provided a rough surface for adherence. Each dish received 2.25 ml of broth and was incubated at 36° in 5% CO_2 and air. The ciliary action of the epithelial cells could be observed through the floor of the dish by using an inverted microscope at 100X magnification. The organ cultures were infected by adding 0.25 ml of the appropriate mycoplasma species obtained from a culture in log phase; 0.25 ml of sterile broth was added to the control cultures.

Histopathology. At the termination of each experiment the tracheal rings were removed

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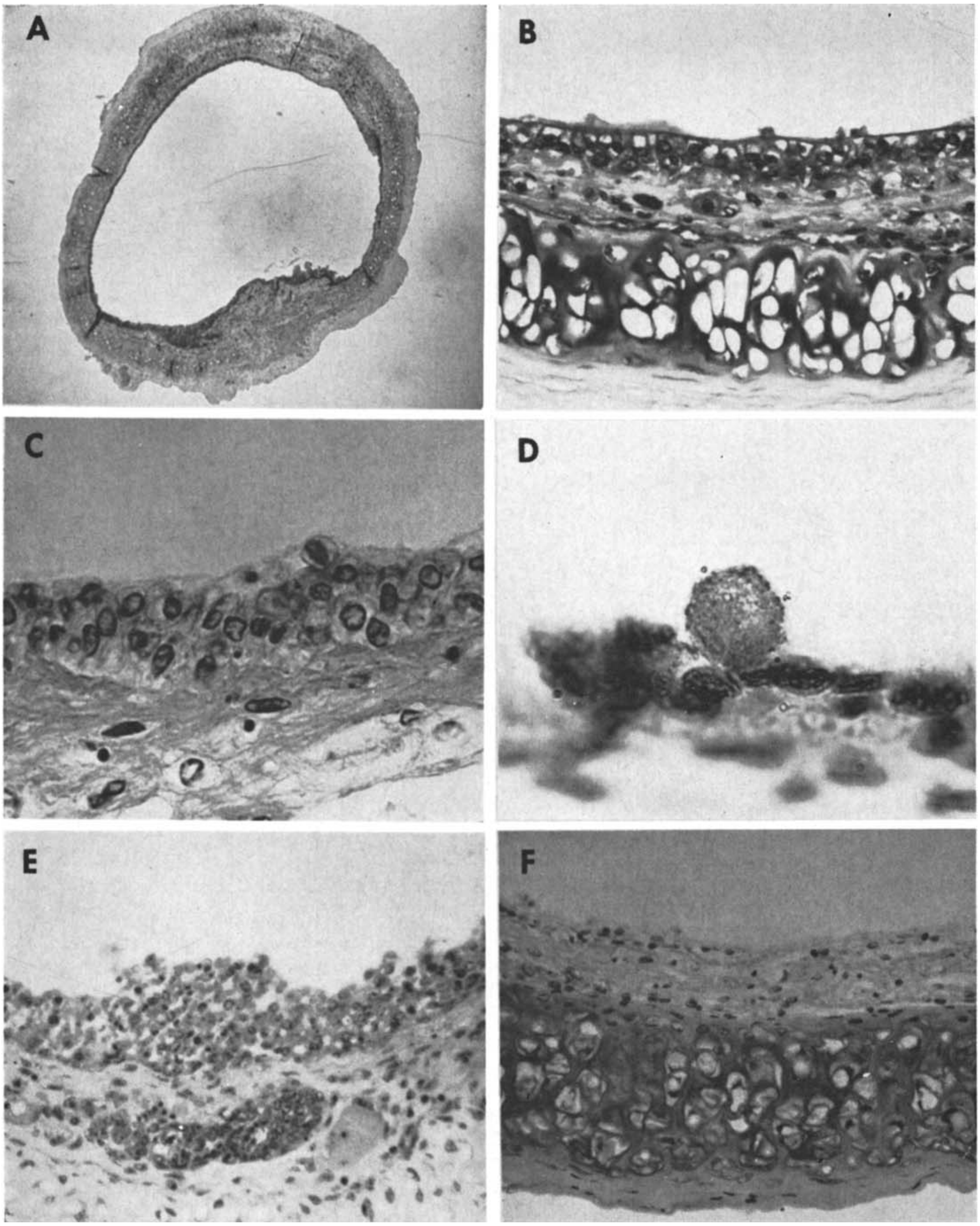

FIG. 1. Hamster trachea infected with *Mycoplasma pneumoniae* (hematoxylin and eosin stain unless otherwise specified). A. Transverse section of normal trachea maintained in organ culture, showing architectural preservation including the ciliated epithelial layer. × 28. B. Tracheal epithelium showing cytoplasmic eosinophilia and vacuolization, 24 hr postinfection. × 238. C. Nuclear swelling and chromatin margination in epithelial cells, 48 hr postinfection. ×595. D. Spherical mycoplasma microcolony attached to tracheal epithelium, 48 hr postinfection. Brown and Brenn technique, × 1,116. E. Loss of cilia and disorganization of surface and submucosal glandular epithelium, 72 hr postinfection. × 288. F. Complete loss of tracheal epithelium, 96 hr postinfection. × 238.

TABLE I. Effect of Virulent *Mycoplasma pneumoniae* on Hamster Trachea *in Vitro*: Influence of Inoculum Size on Time Required for Ciliary Inactivation.

Organism concentration CFU/ml ($\log_{10}$)[a]	Ciliary activity (day)[b]							
	1	2	3	4	5	6	7	8
6.15	+	+	—	—	—	—	—	—
5.15	+	+	+	—	—	—	—	—
4.15	+	+	+	+	—	—	—	—
3.15	+	+	+	+	+	—	—	—
2.15	+	+	+	+	+	+	—	—
None	+	+	+	+	+	+	+	+

[a] Initial colony-forming units in culture fluid.
[b] Days of observation, ciliary motion present (+) or absent (—).

from the organ culture dish and placed in Van de Grift's fixative for 8–18 hr. After fixation the tissue was transferred to 70% ethyl alcohol for at least 4–6 hr, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (see Fig. 1A). Limited use was also made of the Brown and Brenn staining method and the indirect immunofluorescent reaction as previously dscribed (5).

Results. To determine the effect of *M. pneumoniae* on tracheal organ cultures, conditions were established first which would permit maintenance of the tissue as well as propagation of the organism. It was found that tracheal slices remained viable, as indicated by continuation of organized ciliary activity, for as long as 5 weeks when Hayflick's medium was used as the fluid phase and changed every 4–5 days. In organ cultures inoculated with large amounts of virulent *M. pneumoniae* [10^{6-7} colony-forming units (CFU)] the ciliary activity became disorganized and disappeared within 48–72 hr of incubation. To explore the dynamic aspects of this phenomenon experiments were designed to determine the effects of organism quantity and growth phase on the tissue reaction; histopathologic study of the tissue was also conducted.

Evaluation was made first of the effect of mycoplasma quantity on the time required for disappearance of ciliary motion. A series of separate organ cultures was established containing tracheal slices from the same hamster; 10-fold dilutions of an organism pool having $10^{7.15}$ CFU/ml were used as inocula for individual organ culture dishes. Control cultures received sterile broth. At daily intervals the tissues were examined microscopically to determine the presence or absence of ciliary activity. Table I indicates results from a representative experiment. Cessation of ciliary activity was observed after 2 days in the culture vessels receiving the maximum inoculum while 4 additional days were required when 10,000-fold fewer organisms were used. Thus, the time required for disappearance of ciliary activity was related inversely to the initial number of organisms placed in the cultures.

Additional experiments were performed to determine if ciliary interference was related directly to mycoplasma infection or resulted indirectly from changes in the culture system secondary to organism proliferation. Tracheal sections from the same hamster were placed in a petri dish with sterile broth. At daily intervals one of the sections was transferred to second vessel in which a *M. pneumoniae* culture had been established, containing initially 10^6 CFU/ml; companion sections were transferred to a third vessel containing sterile broth at corresponding intervals to serve as controls. The infected and control dishes were incubated and examined daily for ciliary activity. In the experiment illustrated in Table II, the section placed in the culture initially lost ciliary activity after 48 hr, while a section introduced on day 3 showed good activity for 6 days. No changes occurred in sterile control cultures. The increasing duration of ciliary motion seen in

TABLE II. Effect of Virulent *Mycoplasma pneumoniae* on Hamster Trachea *in Vitro*: Influence of Organism Growth Phase on Time Required for Ciliary Inactivation.

Tracheal sections[a]	Age of mycoplasma culture, days[b]										
	0	1	2	3	4	5	6	7	8	9	10
A	[c]	+	+	—	—	—	—	—	—	—	—
B		[c]	+	+	+	—	—	—	—	—	—
C			[c]	+	+	+	+	—	—	—	—
D				[c]	+	+	+	+	+	+	—

[a] Maintained in sterile culture until transfer to inoculated dish.
[b] Ciliary motion present (+) or absent (—).
[c] Day section transferred.

sections newly added to the aging culture suggested that the tissue injury was related to direct infection or contact with viable organisms. If this effect were the result of accumulated metabolic by-products or medium depletion, damage would be expected to occur more rapidly rather than after successively longer intervals as new tissue was added.

The preceding experiments employed virulent *M. penumoniae* as inoculum. The specificity of the observed interference with ciliary activity was evaluated by establishing tracheal organ cultures as before which were inoculated with four other human mycoplasma species; in addition, the effect of an attenuated *M. penumoniae* strain was examined. Table III indicates initial and final organism concentration in each culture, and observations of cilia over a 6-day period. No apparent effect on the ciliary activity was seen in cultures inoculated with *M. fermentans, M. hominis, M. pharyngis,* or *M. salivarium.* Growth of these strains was evident microscopically in the culture vessels, by pH changes of the media, and by the residual organism titers at termination of the experiment. In contrast to the expected effect of virulent *M. pneumoniae,* the attenuated strain did not cause interference with ciliary motility until day 6 when growth had produced marked acidity. This suggests that the hamster tracheal organ culture may be a useful tool for distinguishing virulent and attenuated *M. pneumoniae* strains. The results also differentiate *M. peumoniae* from four other human mycoplasma species which are thought to be non-pathogenic.

Histopathology. To determine the morphologic basis of interference with ciliary activity produced by virulent *M. pneumoniae,* microscopic study was made of tissues utilized in the experiments described. Specimens lacking apparent ciliary activity were found to have disorganization or loss of the epithelial layer. Sequential changes preceding this event were defined by histologic examination

TABLE III. Effect of Five Human Mycoplasma Species, Including Virulent and Attenuated *Mycoplasma pneumoniae* Strains, on Ciliary Activity of Hamster Trachea *in Vitro.*

Mycoplasma species	CFU/ml,[a] $\log_{10}$ (day)		Ciliary activity[b] (day)					
	0	6	1	2	3	4	5	6
M. fermentans	7.3	6.6	+	+	+	+	+	+
M. hominis	7.2	5.0	+	+	+	+	+	+
M. pharyngis	7.7	6.5	+	+	+	+	+	+
M. salivarium	7.8	6.6	+	+	+	+	+	+
M. pneumoniae (virulent)	6.0	7.3	+	+	+	—	—	—
M. pneumoniae (attenuated)	6.2	7.2	+	+	+	+	+	—

[a] Colony-forming units/ml of fluid in culture vessel at time indicated.
[b] Ciliary activity present (+) or absent (—).

of tissues infected for varying periods, as illustrated in Fig. 1. The earliest alteration, occurring within 24 hr, was increased cytoplasmic eosinophilia; this was followed by cytoplasmic vacuolization (Fig. 1B), and nuclear swelling with chromatin margination (Fig. 1C). Subsequently cilia were lost, associated with the appearance of hemotoxylin-positive material in clumps at the cell surface (Fig. 1D), and disturbance of cellular polarity and organization which was also noted in epithelium of submucosal glands (Fig. 1E). In later specimens, nuclear and cytoplasmic disruption was seen, with eventual loss of the epithelial layer (Fig. 1F). These changes appeared to be associated specifically with virulent *M. pneumoniae* infection, since they were absent in sterile control specimens, and in tracheal cultures infected with other mycoplasma species (see Table III).

The extracellular hematoxylin-positive material shown in Fig. 1D resembled *M. pneumoniae* colonies described in the hamster, both morphologically and by staining reaction with the Brown and Brenn method (5). This material was identified as *M. pneumoniae* by bright fluorescence after application of specific rabbit antiserum and fluorescein isothiocyanate-conjugated goat anti-rabbit globulin. No fluorescence was seen in uninoculated tissue similarly treated, or in infected sections treated with normal rabbit serum.

Discussion. The pathogenesis of *M. pneumoniae* disease is poorly understood, in part due to limited information on the organism's biology and to the low mortality of natural infections preventing pathologic insight. This has necessitated the application of experimental models to analysis of the host–parasite interaction. Tracheobronchitis is the most frequent clinical expression of human *M. pneumoniae* infection, suggesting that the respiratory mucosa is intimately involved in the disease process. Animal models provide the specialized mucosal cells needed for study of the host–parasite relationship at the cellular level, but analysis is complicated by lack of direct control over the local environment. Differentiated epithelial cells cannot be maintained in conventional tissue culture, but this problem can be circumvented by use of organ culture systems.

Ciliated respiratory epithelium in organ culture has been used for study of viral agents by Hoorn (3) and others. In tracheal segments the presence of the agents is indicated by ciliostasis, cellular inclusions, or viral particles detected by electron microscopy (8). The present study was undertaken to investigate the feasibility of tracheal organ culture as a model for *M. pneumoniae* disease, and to assess the effects of the organism on the mucosal cells. Use of organ cultures for study of mycoplasma infections, and establishment of hamster tracheal cultures, have not been reported previously.

The system described in the present report provides a source of differentiated target cells in the form of ciliated respiratory epithelium that may be maintained, observed, and manipulated readily. The results of this study indicate unique abilities of *M. penumoniae* to produce tissue injury, in comparison to lack of apparent alterations associated with other human mycoplasmas examined. The physiologic and anatomic alterations occurring in response to *M. pneumoniae* infection indicate that the model may be useful for dissection of pathogenetic mechanisms. Although it has been postulated by others (9) that the cellular attachment mechanism and peroxide secretion of *M. pneumoniae* may be important in pathogenicity, no differences in these properties were demonstrated in another study from this laboratory between strains classified as fully virulent or attenuated on the basis of pathogenicity testing in the hamster (6). In the present experiments the attenuated (Mac) strain could be differentiated from the virulent strain in that there was diminished evidence of tracheal epithelial damage. These observations suggest the existence of additional mediators of virulence in *M. pneumoniae.*

The pathogenic significance of human mycoplasmas other than *M. pneumoniae* is unknown; it may prove feasible to investigate the potential of other species to produce tissue injury using appropriate organ culture models in situations where disease associations have been suggested. For example, urinary tract mucosal cells could be infected with *M. hominis,* or fetal tissues with T-strain mycoplasmas, to assess the effect of these organisms on differentiated specialized cell types not available with conventional tissue cultures.

Limited information is available on the sequential response of the ciliated epithelium to injury by pathogens of the respiratory tract. The effect of the influenza virus on the respiratory epithelium has been studied more extensively than any other pathogen. Walsh *et al.* did tracheobronchial mucosal biopsies on six patients with type A, Asian strain influenza uncomplicated by bacterial infection (10). A spectrum of changes was described ranging from vacuolization, edema, and loss of cilia in epithelial cells to desquamation of the mucosa down to the basement membrane. These findings are similar to the response of the hamster trachea in organ culture infected with *M. pneumoniae*; preliminary experiments also suggest that similar results can be obtained with human embryonic trachea. Whether this spectrum of changes seen in the ciliated respiratory epithelial cells is a general response to injury elicited by many different respiratory pathogens or is specific to only a few is not known.

Summary. The applicability of organ culture systems to the study of mycoplasma infections is illustrated by the experiments described. Tracheal organ culture provided a controllable environment for analysis of the interaction between *M. pneumoniae* and the natural target cell of this pathogen, the ciliated respiratory epithelium. *Mycoplasma pneumoniae* produced distinct cytopathology in this system that was not seen with other human mycoplasmas tested. In addition, virulent *M. pneumoniae* produced tissue damage more effectively than did an attenuated strain. The observations suggest that this experimental model may be useful for the analysis of factors contributing to organism pathogenicity.

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