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Attachment of *Mycoplasma pneumoniae* to Tracheal Monolayer Outgrowths

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Mycoplasma pneumoniae is a human pathogen of the respiratory tract. It attaches to the ciliated respiratory epithelium by means of its attachment tip and the sialoglycoprotein receptor site on host cells. To study the mechanical and biochemical features of the attachment process, we developed a new *in vitro* biological model of respiratory tissue. The ciliated monolayer system involved a collagenase treatment of rodent tracheal explants, followed by incubation in Waymouth's MAB 87/3 medium. Epithelial migration led to the development of patches of cell monolayers both interior and exterior to the tracheal lumen. After seven days of incubation, monolayer patches contained 20 percent ciliated cells. Ciliary motion was active for several days after the explant was removed. When exposed to *M. pneumoniae* for two hours, the ciliated cells became covered with pathogen. The mycoplasmas were in close association with the host cell membranes, and could lie horizontally along the membrane when not physically held in a vertical orientation by cilia. Cytonecrosis developed within 48 to 72 hours.

INTRODUCTION

Mycoplasma pneumoniae is one of the agents which can cause primary atypical pneumonia (PAP) in humans [1]. The disease is typically non-fatal and involves the conducting airways. It can cause considerable discomfort and seriously reduce activity in affected persons (normally children and young adults). Recently, there has been considerable interest in mycoplasma-associated PAP due to an increase in sequelae and complications. These range from skin rashes [2] to several types of disturbances in CNS function, including paralysis [3].

M. pneumoniae exerts its pathogenic effect through an (as yet) undefined mechanism. We do know, however, that the initial, key event in the infection process is the interaction of the mycoplasma with a receptor site of specific, biochemical composition on the surface of respiratory epithelial cells [4]. This is only one type of possible attachment, since *M. pneumoniae* also can attach in an electrical charge-mediated fashion to inanimate surfaces such as glass and plastic.

The fact that a receptor site is involved in the cytotoxic infective process is not surprising, since all pathogens which infect epithelial cells have to deal with numerous defense mechanisms of the host. All epithelial surfaces are characterized by being (*a*) in a flowing environment in which gas, liquid, or semisolids create a physical force which tends to dislodge particles from the surface; and (*b*) constantly renewed as "old" epithelial cells dislodge themselves and are replaced by newly differentiated cells arising from a basal layer. In the case of *M. pneumoniae,* it must not only pass through several microns of flowing mucus and pericellular fluid, but it also must

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withstand the continual beating of cilia. The majority of the epithelial cells on several portions of the conducting airways each contain several hundred cilia, beating at approximately 1,200 beats per minute in a coordinated fashion.

In an attempt to understand the physical and biochemical processes involved in the attachment of *M. pneumoniae* to epithelial cells, several biological model systems have been employed. The hamster and guinea pig serve as the preferred *in vivo* models [5]. Their use by several laboratories around the world has led to new knowledge on the significant and complex role of the host immune system in the gross lesions of atypical pneumonia. However, studies on the details of the interaction between individual parasite and host cells have concentrated on *in vitro* models [6]. These are isolated from hormonal and physiological influences which conceivably might alter the interaction adversely. In addition, environmental variables and the atmosphere which impinge on the cells can be controlled with greater precision in the *in vitro* models.

In vitro cellular systems are available with various degrees of complexity. Each has both advantages and disadvantages. Isolated, perfused organ culture of intact rodent trachea was introduced as a model for *M. pneumoniae* infection last year [7]. It is especially useful for distribution studies of pathogens along the airway surfaces. Explant culture of sections or transverse slices ("rings") of trachea and bronchi have been used quite successfully for several years to examine the consequences of infection on the host cell [8]. However, both of these models have surfaces lined with several cell types, and the dense lawn of ciliary strands does not permit a morphological analysis of the surface of individual ciliated epithelial cells. In an attempt to create an opportunity to study the details of the interaction between *M. pneumoniae* and ciliated cell membranes, we have developed a ciliated outgrowth culture system [9,10]. The object was to maintain these highly differentiated cells in a monolayer format.

MATERIALS AND METHODS

Preparation of the ciliated monolayers begins with the aseptic excision of an intact trachea from a susceptible rodent such as a hamster or guinea pig. Transverse slices are then made to create ring explants. After being rinsed, these are immediately placed in a solution of 0.01 percent collagenase (Gibco, Grand Island, NY) in roller tubes for approximately 60 minutes [9]. This very gently loosens the connective tissue matrix which supports the highly organized epithelial layer. When these rings are placed on glass coverslips under a thin layer of tissue culture medium (for example, Waymouth's MAB 87/3 with 10 percent fetal calf serum; Gibco), the natural epithelial outgrowth or migration is greatly enhanced. After four to six days, zones of epithelial monolayer growth become evident. These tend to fill the lumen of the ring explant and extend several millimeters out from the tissue of origin (see Fig. 1, reproduced from [10] with permission). Most of the ciliated cells are in the lumen, but numerous ciliated cells also migrate out of the ring as the cut surfaces are "repaired" biologically. Each explant will give rise to several thousand monolayer cells in five days. An average of 20 percent of them are ciliated. After the "parent" explant is removed, the ciliated cells in the lawn will continue to beat for three to seven days. Each of the explants can then be placed on a new coverslip and will create new patches of monolayers. Each of the "crops" has outgrowth efficiency lower than the previous one, but one to three passages will give quite usable zones of growth.

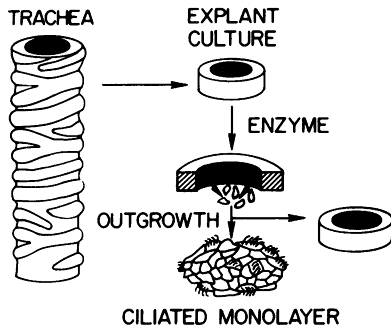

FIG. 1. Diagram of the process used to prepare ciliated monolayer cultures from tracheal epithelium. (Reproduced with permission, [10]).

Much of the early work was done on glass coverslips since they are easy to handle and work well with the metal coating steps required for scanning electron microscopy. Recently, we also have prepared the monolayers on gas-permeable teflon membranes [10]. Membranes are clamped in a special device [10] known as a Bionique Chamber/Dish (Corning Glass Works, Corning, NY). The etched teflon membrane is permeable to oxygen, and cell attachment is equivalent to that of glass. The membrane serves as both the growth substrate and the bottom surface of the vessel, so high-resolution, optical microscopy can be used to follow the development of the outgrowth.

The main advantage of this approach is the ease of doing electron microscopy studies. Samples can be fixed, stained, and dehydrated *in situ*. For transmission EM of monolayers with mycoplasmas attached, embedding medium can be placed directly in the chamber. Once it has polymerized, sections can be cut through the thin teflon membrane. Alternatively, the teflon can be pulled off the plastic with adherent cells. Blocks can then be cut, re-embedded, and sectioned. In order to prevent the membrane from stretching and buckling during the embedding process, the Chamber/Dish was placed on a teflon spacer (Corning Glass Works, Corning, NY) 1 cm high and 1 cm in diameter. This kept the membrane taut and flat, so that after the resin hardened, there was a 1 cm zone which was smooth and regular. When the plastic embedding medium was then removed and cut, one could easily discern the flattened area which has all of the cells in the same plane. We have done both vertical and en-face sectioning of ciliated monolayers infected with *Mycoplasma pneumoniae*. This approach, coupled with scanning electron microscopy, provided a unique opportunity to examine the physical interaction of pathogens attaching to a ciliated host cell membrane.

RESULTS AND CONCLUSIONS

Scanning electron microscopy studies reveal that the ciliated cells are often located in the same area. These zones can be surrounded by non-ciliated fibroblasts and epithelial cells, but normally are in contact with the edge of the explant. The arrangements of cilia on monolayer cells are diverse. They can be in clusters or in long rows (Fig. 2). The actual physical organization apparently depends on the shape of the cell as it was positioned in the pseudostratified epithelial layer.

In the process of being fixed, dehydrated, and metal-coated, cracks often develop along the cell boundaries. These serve to indicate the area filled by individual cells. Membranes are quite evident in contrast to the cilia-covered view of the normal

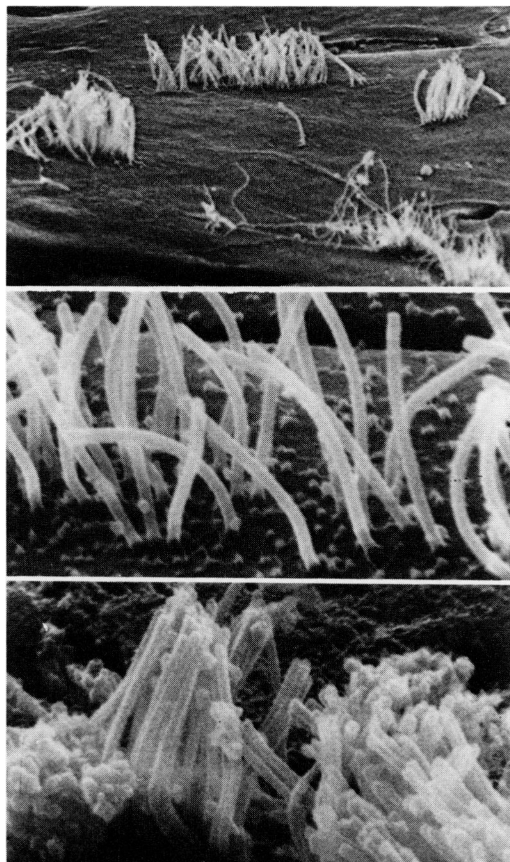

FIG. 2. Scanning electron micrographs of ciliated monolayer outgrowths (top to bottom: 2,000/10,000/10,000 ×). Lowest photograph shows mycoplasmas in infected culture.

trachea surface. At times, the intercellular boundaries remained tight, but individual ciliated cells could still be seen due to a ridge which ran along the outer boundary of the cell membrane. In addition, the surface of the ciliated cell was often (though not always) covered with an extensive array of microvilli. As has already been mentioned, the cilia continued to beat actively in this configuration.

We have developed an electronic video system based on a frequency counter and motion detector for quantitating ciliary activity. The beat frequency ranges from approximately 10 to 25 beats/second, and is very similar to that noted in tracheal explants. However, it was observed that not all of the ciliated cells beat at the same frequency. There was a significant variance detected in the frequency of ciliated monolayer cells in the same culture. This suggested a lack of coordination such as that seen in organized respiratory tissues. We also observed that cilia in different areas on the same cell can beat at different frequencies.

The real advantage of this monolayer technique is that one can observe the host cell close up (Fig. 2). One can even count the cilia per cell, analyze the size and shape of individual ciliary strands, and study the morphology of the cell membranes.

When suspensions of mycoplasmas were added to such outgrowths, the mycoplasma readily attached. Attachment was receptor site-mediated, as evidenced by the fact that neuraminidase pretreatment reduced attachment of *M. pneumoniae* by 75 percent. In a given cell suspension, approximately 10 percent of the

mycoplasmas were capable of attachment. Mycoplasmas were well distributed over the epithelial cell membrane, including the cilia (Fig. 2). If located more than a few microns away from the cilia, they were found to lie along the cell membranes. There did not appear to be any preferential distribution along any given cell. Mycoplasmas were normally attached via the constricted end known as the attachment site. This differentiated tip structure, a specialized organelle of *M. pneumoniae,* is best seen in sections viewed with transmission electron microscopy (Fig.3).

The tip was observed in coccobacillary forms, but was most prominent in filaments one or more microns in length. It occurred only on one end, and was characterized by an electron-translucent space enclosing a dense, electron-opaque core or shaft. The end with the tip was normally somewhat constricted. Hence, the attachment tip also could be discerned with scanning electron microscopy.

The mycoplasma attachment tip could readily be observed in close association with monolayer host cell membranes, including the cilia over which the cell membrane extends. When an *M. pneumoniae* organism was lodged in between two cilia, the attachment tip was clearly oriented toward the cell surface. On cells which contained large microvilli, the mycoplasmas could also be seen in between microvilli and/or cilia. The highest frequency of attachment tips is noted along the host cells. Once the mycoplasmas were oriented a short distance from external appendages, their orientation was not necessarily vertical (Fig. 3). This figure also illustrates a tendency for overlap between the eucaryotic cells. As in many (or perhaps most) monolayer systems, contact inhibition is not an absolute phenomenon. In reality, cell edges overlap and even interdigitate, though in a light microscopic examination of cultures the cell borders seem quite distinct and separate.

If one views a reasonably smooth cell surface with few cilia or microvilli, the mycoplasmas can be seen to lie horizontally along the membrane. This is the same configuration we noted previously in non-ciliated lung fibroblast monolayer systems [12,13]. In the ciliated monolayer explant system, we have seen both vertical and horizontal orientations to the mycoplasmas.

Some of the most intriguing views are those that suggest physical alterations at the juncture of host and parasite membrane. There are several instances in which the

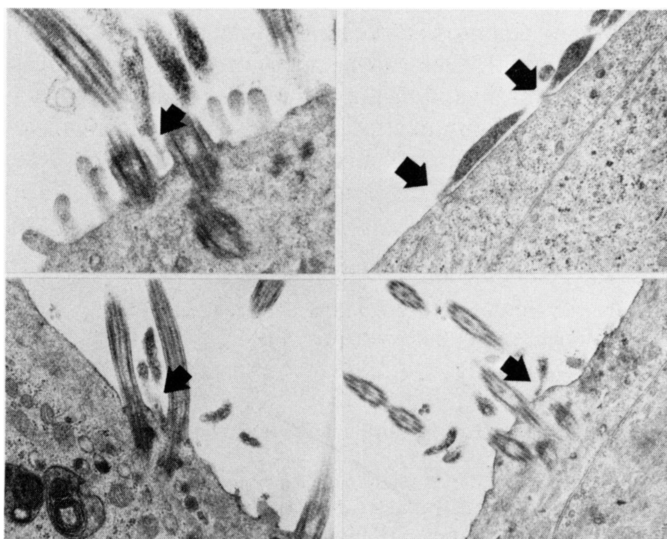

FIG. 3. Transmission electron micrographs of *M. pneumoniae* (*arrows at attachment tips*) on ciliated monolayer cells (*upper left:* 13,000 ×, remainder at 11,000 ×).

boundaries at the membrane contact point become indistinct. While such a view cannot prove the existence of a fusion-type event, it is definitely suggestive of some form of intimate membrane association and alteration at the attachment site. Areas of particular interest include the base of the cilia, where the filamentous mycoplasmas are often wedged between the cell appendages. It is quite possible that the physical action of the beating cilia may even facilitate a physical re-alteration of membrane organization once the pathogen has contacted the sialoglycoprotein receptor site and, conceivably, initiated some type of post-attachment chemical reaction. While one must be cautious and conservative in interpreting any type of morphological evidence, it does suggest that further attention be devoted to the biophysical activity below the attachment tip.

The tracheal outgrowth system is clearly a unique type of monolayer. As such, it offers several advantages for the study of host-parasite interactions such as those which involve mycoplasmas. Typical monolayers contain undifferentiated cells which do not precisely mimic, either morphologically or metabolically, those from the natural target tissue. The ciliated outgrowth system described here provides highly differentiated cells identical to those involved in *in vivo* infections. Another advantage is that one can observe the membrane of viable, ciliated cells. This is virtually impossible in explant cultures because of the dense packing of the cilia on the exposed surface.

While classical monolayers (e.g., lung fibroblasts as in [4]) often contain receptor sites specific for mycoplasmas, they may not be identical biochemically to those involved in actual disease production. However, these undifferentiated monolayer cultures do have the dual advantage of homogeneity and potential for mass culture. Conversely, these are two drawbacks to the outgrowth system: the patchs of outgrowth are limited is size, and contain 20 percent ciliated cells and 80 percent non-ciliated cells such as goblet cells, fibroblasts, and other epithelial basal cells. All *in vitro* models represent a compromise. In this instance one sacrifices quantity and homogeneity for membrane exposure, functionality, and an accurate representation of host cell type.

The data just reviewed illustrate the utility of the ciliated monolayer outgrowth technique for the study of mycoplasma infections. It is not limited to mycoplasmas, however, since Dr. Ronald Mink in our laboratory has also used this model to study infection of respiratory epithelium by *Chlamydia trachomatis,* the cause of newborn pneumonitis [manuscript in preparation]. One can also use this model system to study the basic biology of epithelial cells, including highly specialized structures such as cilia and desmosomes. When used in conjunction with objective, biochemical-type assays, this new model system will be capable of contributing to our knowledge base on host-parasite interactions in a most significant fashion.

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