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Injury of Respiratory Epithelium

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The large surface area provided by the respiratory tract epithelium of humans for exposure to microbial agents and toxic substances in the environment makes this organ system very vulnerable but a good early indicator of adverse health effects. However, the complexity of pulmonary defense mechanisms complicates definition of the interactive effects of pollutants and infectious agents.

Tracheal organ culture has been utilized to maintain organized, differentiated respiratory epithelium *in vitro*. This model system permits the exposure of respiratory epithelium to injurious agents in an easily visualized and controlled environment. Effects of individual toxin and/or infectious agents may be examined without the involvement of most host defenses and unwanted secondary microbial invaders which hamper interpretation of *in vivo* model studies. Further, elements of host immune response, pharmacologic agents and the like may be added selectively if desired.

A body of information is being developed on specialized respiratory cell injury by various common pathogenic agents — including respiratory syncytial virus, parainfluenza virus type 3, *Bordetella pertussis* and *Mycoplasma pneumoniae* — through studies in tracheal organ cultures. These agents injure specialized epithelial cells in different ways, providing a spectrum of changes against which the added effects of toxic substances could be evaluated at the cellular and subcellular levels. Information on the pathogenesis of infectious/toxic injury could suggest new directions for human health research and for means to benefit the human host.

Introduction

The respiratory tract of humans provides a large surface area that is constantly in contact with the environment. The lower respiratory tract is normally sterile, emphasizing the remarkable efficiency of the defense mechanisms of this organ system. At the present time little is known about the effects produced by inhalation of foreign particulates and infectious agents on the epithelial lining of the respiratory tract. It is known that for the respiratory tract to function properly the alveolar macrophages and elements of the mucociliary escalator must be intact. Once these defense mechanisms have been altered, a build-up of both viable and nonviable inhaled substances occurs on the epithelium which may jeopardize the health of the host.

The anatomic and physiologic complexities of the respiratory tract have presented problems in interpreting the effects of exogenous agents on this organ system, since the data base on normal function has not been well delineated. This has led us to establish less complex *in vitro* respiratory organ culture models that can be more easily observed under controlled conditions. In this paper, studies will be reviewed in which tracheal organ culture has been utilized to examine the pathogenic potential for epithelial injury by *Mycoplasma pneumoniae, Bordetella pertussis,* respiratory syncytial virus, and parainfluenza virus type 3. The manner in which these human respiratory pathogens injure respiratory epithelium in tracheal organ culture will be contrasted.

Tracheal Organ Culture Model

Tracheas removed from most animals and human fetuses are suitable for study in this model system (*1, 2*). The organ culture is prepared by removal of the trachea by a sterile technique. After making a midline incision in the anterior neck, the trachea is exposed and transected at the levels just below the larynx and above the carina. The trachea is then cut into rings with a scalpel. Each ring contains a cartilage which provides support. The rings of tissue are placed on areas of cross-hatch scratches in a small plastic petri dish and enough tissue culture medium added to just cover the rings. The culture is then maintained at 36°C in 5% CO_2 and air.

The inner surfaces of the tracheal rings are lined by organized, differentiated, respiratory epithelium that can be maintained up to six weeks when the

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medium is changed every three days. At any time, the dish may be removed from the incubator and the epithelium examined using an inverted microscope without opening the petri dish. This permits one to examine for the presence and vigor of beating of the cilia which serves as a good indicator of viability of the epithelium and degree of injury produced by test substances.

One trachea provides 13-15 rings, thus permitting tissue with the same genetic make-up, for test and control dishes. This model is very maneuverable, in that rings of tissue may be added or removed from a dish at any time under sterile conditions. The rings can be grasped on their outer surface by forceps and moved without touching their epithelial inner surface.

The animal may be exposed to the test substance prior to sacrifice and removal of the trachea for organ culture; or, the tracheal rings may be prepared and then exposed to the test substance. In our laboratory we have had more experience with the latter method.

Mycoplasma pneumoniae in Hamster Tracheal Organ Culture

Mycoplasma pneumoniae is the most common cause of pneumonia in teenagers and young adults. This microorganism is the smallest infectious agent capable of growth on lifeless media. In an attempt to better understand the pathogenesis of disease produced by this microorganism, hamster tracheal organ cultures were inoculated with both virulent and attenuated strains of *M. pneumoniae* and other human mycoplasma species that produce no known disease in man (*Mycoplasma fermentans, Mycoplasma hominis, Mycoplasma orale,* and *Mycoplasma salivarium*). The virulent strain of *M. pneumoniae* produced ciliostasis by day 3 and the attenuated strain by day 6. The other strains of mycoplasmas had no effect on ciliary activity implying that the ciliostatic property possessed by virulent *M. pneumoniae* might play a role in the virulence of this respiratory pathogen (*3*).

In order to be able to quantitate the ciliary beat frequency, a calibrated stroboscope was used as the light source for the inverted microscope (*2*). The speed of the ciliary beat could be determined from the flash frequency which gave the illusion of arrested ciliary motion. A series of separate cultures was established containing tracheal rings from the same animal; tenfold dilutions of the virulent *M. pneumoniae* organism pool were employed as inocula. The control rings were placed in sterile broth. Cessation of ciliary activity was produced within four days by the two greatest inocula, while the smallest inoculum required seven days to reach this point. This provides evidence of a dose response effect and suggests that a critical degree of infection is required for tissue injury to occur.

Individual tracheal rings can be removed from the culture dish at any time and examined by microscopy techniques. When tracheal rings fixed with formalin and stained with hematoxylin and eosin were examined, there was good preservation of the ciliated epithelium in the control rings after 48 hr in organ culture. In contrast, the epithelium of comparable tracheal rings infected for 48 hr in organ culture with *M. pneumoniae,* demonstrated nuclear swelling with chromatin margination, cytoplasmic vacuolization, and loss of cilia from some epithelial cells (*4, 6*).

In an attempt to localize the *M. pneumoniae* organisms in relationship to the injured cells, tracheal rings were removed from the organ culture and frozen sections prepared. These sections were treated with rabbit *M. pneumoniae* antiserum followed by goat antirabbit globulin conjugated with fluorescein isothiocyanate. When the *M. pneumoniae* infected tissue was examined with an immunofluorescent microscope, the antigen was found to be located along the lumenal border of the epithelial cells. In tissue that had been infected for 72 hr, clumps of specific antigen were seen in the lumen and down into the epithelium on each side of the non staining nuclei. Control tissue demonstrated no specific fluorescence. Since the *M. pneumoniae* were below the resolution limits of the immunofluorescence microscope, it was not possible to delineate their exact location in respect to the epithelial cells by this technique (*4*).

In order to answer this question, the tracheal rings were examined by electron microscopy. Tissue was removed from the organ culture dish, fixed in glutaraldehyde and osmium tetraoxide, embedded in epon and thin sections cut. When tracheal rings infected for 48 hr were examined, densely staining filamentous organisms were seen down between the cilia. At higher magnification the mycoplasma organisms were seen to attach to the epithelial cell by a specialized tip structure. The tip structure consisted of a dense central filament surrounded by a lucent space which was enveloped by an extension of the organism's unit membrane. The outer membranes of the microorganism and mammalian cell were in close approximation, but were not fused (*2, 3*).

On further examination *M. pneumoniae* were frequently seen attached in the region of the terminal bar where the lumenal surface of individual epithelial cells were joined. Organisms were also seen penetrating the intercellular spaces and multiple organisms forming microcolonies were seen in intercellular spaces between epithelial cells (*2*).

Tracheal rings infected for 72 hr revealed the pathologic changes of both epithelial cell cytoplasmic vacuolization, and loss of cilia. *M. pneumoniae* organisms were seen in the intercellular spaces but no organisms were seen within the epithelial cells. This explained the findings seen with immunofluorescence and the thicker frozen sections. The antigen was present in the intercellular spaces (*3*).

Another technique that we have utilized to examine the tracheal organ culture model has been that of scanning electron microscopy (*5*). This method of observation permits an in depth look at the lumenal surface of the epithelial layer. Numerous filamentous *M. pneumoniae* organisms were seen attached to the lumenal border, and the apical portions of infected ciliated cells were seen sloughed into the lumen. The phenomenon, called ciliocytophthoria, was found to be the characteristic pattern of epithelial cell injury produced by *M. pneumoniae* infection.

The tracheal organ culture model permits the examination of the biochemical basis of the alterations produced in epithelial cell injury by infectious agents (*6-8*). Exposure of hamster tracheal rings to virulent *M. pneumoniae* organisms in organ culture leads to alterations in macromolecular biosynthesis and metabolic activity of the respiratory epithelial cells prior to the occurrence of histopathologic changes. These changes were not seen with avirulent organisms derived from the same parent strain. Within 24 hr after infection of tracheal rings by virulent *M. pneumoniae*, inhibition of host cell ribonucleic acid and protein synthesis was evident. The addition of erythromycin 24 hr or earlier after infection prevents the onset of abnormal orotic acid uptake and subsequent cytopathology. However, 48 hr after infection, rescue of host cells by erythromycin does not occur and pathological changes become evident.

Our data indicate that the intimate contact between virulent mycoplasmas and the respiratory epithelium alone does not account for the subsequent interruption of host cell metabolism but must be accompanied by continued multiplication and biochemical function of attached mycoplasmas. The mechanism of injury mediation is not yet known but the primary effect of mycoplasma infection on tracheal organ culture may be at a transcriptional or translational level.

Mycoplasma pneumoniae in Human Fetal Tracheal Organ Culture

Human fetal trachea in organ culture has also been utilized to study the epithelial changes produced by *M. pneumoniae* (*9, 10*). The ability of mycoplasma species to produce ciliostasis was very similar to that reported above in hamster tracheal organ culture. Only virulent *M. pneumoniae* injured the human tissue. When examined by light microscopy, the epithelial changes produced by *M. pneumoniae* followed the attachment of the organisms between the cilia. This was followed by the apical portion of the cell becoming eosinophilic and protruding into the lumen with the cilia forming a sunburst pattern. The ciliated apical portion of the cell was later sloughed into the lumen with the basal portion of the cell being left behind to maintain the epithelial barrier.

Immunofluorescence studies, as previously described with tissue from the hamster tracheal organ culture system, showed the *M. pneumoniae* antigens to be localized on the epithelial lumenal border between the cilia of the human tissue. The organisms were also seen to invade the submucosal glands.

When the human tracheal tissue was examined by electron microscopy, the *M. pneumoniae* organisms were seen to attach to the lumenal aspect of the cell by their specialized tip structure. No evidence of organisms entering the epithelial cells was seen. The ciliocytophthoria pattern of epithelial cell injury produced by *M. pneumoniae* was also observed at the electron microscopic level. The lumenal portion of the ciliated cell first protruded into the lumen and was followed by a constricting and pinching off of the apical surface. The injury produced by *M. pneumoniae* may be due to parasitization of individual cells, since heavily parasitized cells showed marked damage of subcellular organelles, and adjacent less heavily parasitized cells appeared normal.

Bordetella pertussis in Hamster Tracheal Organ Culture

The hamster tracheal organ culture model has been employed to examine the effects of *B. pertussis* on respiratory epithelium (*11*). This human bacterial pathogen is the causative agent of whooping cough in children. When the tracheal rings were infected with phase I *B. pertussis* and examined by light microscopy, the bacteria were seen attached to the ciliated cells; but, unlike *M. pneumoniae*, there was no attachment to the nonciliated cells. After attachment, the ciliated cells were injured and later extruded from the epithelial layer. The extruded cells were seen to contain pynotic nuclei with bacteria attached only to their lumenal surface. After 72 hr of infection in organ culture, no ciliated cells remained, and the spaces left by the extruded ciliated cells in the epithelial layer had been filled in by the nonciliated

cells. No attachment of *B. pertussis* to the remaining nonciliated cells was observed.

Injury of the ciliated epithelial cells was also examined by electron microscopy. Transmission electron photomicrograph showed ciliated cells that were heavily infected with *B. pertussis*. Some of these ciliated cells were in the process of being extruded from the epithelial layer. These cells possessed markedly damaged subcellular organelles while the adjoining cells appeared normal. At no time were *B. pertussis* organisms seen within the epithelial cells.

We have examined comparable tracheal epithelium infected in organ culture with *B. pertussis* by scanning electron microscopy (*12*). After 48 hr of infection there were multiple extruded cells seen on the lumenal surface. This technique, at higher magnification, also provided a three-dimensional view of parasitized ciliated cells being extruded from the epithelial layer. The ciliated cells that had been extruded had bacteria attached to their lumenal surface and there were marked membrane changes with some appearing as hollow shells with their nucleus having been expelled. No *B. pertussis* organisms were seen attached to nonciliated cells.

Respiratory Syncytial Virus in Human Fetal Tracheal Organ Culture

Respiratory syncytial virus is an important cause of bronchiolitis in young children. The response of the respiratory epithelium in organ culture to infection with respiratory syncytial virus is quite different from those of *M. pneumoniae* and *B. pertussis*. When the epithelium was examined by light microscopy, the first changes seen to develop were perinuclear inclusion bodies in the ciliated cells. This was followed by swelling of the ciliated cells with protrusion into the lumen and loss of cilia. Respiratory syncytial virus infection produced fusion of the infected cell membrane with the cell membranes of adjoining epithelial cells thus forming a giant cell containing multiple nuclei (*13*).

When human tracheal rings are removed from the organ culture after infection and stained with fluorescent antibodies that are specific for the antigens of respiratory syncytial virus, individual cells along the lumenal border are seen to fluoresce. The respiratory syncytial virus was found to infect only ciliated cells in the epithelial layer. No specific fluorescence was seen in cells of the lamina propria or cartilage layer.

Tissue from organ cultures comparable to that just described was processed for electron microscopy and examined. Viral particles were seen budding from the lumenal aspect of the ciliated cells and from the surface of the cilia. The typical perinuclear inclusion bodies produced by respiratory syncytial virus infection of epithelial cells were seen to be made up of a dense intertwining of RNA cores. These cores were later packaged at the membrane of the cell where they formed new viral particles.

Parainfluenza Virus Type 3 in Hamster Tracheal Organ Culture

Another important viral pathogen of children is parainfluenza virus type 3. The epithelial cell injury produced by this virus has been studied in hamster tracheal organ culture (*14*). Thirteen days after infection in the organ culture the formation of giant cells, similar to those seen in the human epithelial tracheal organ culture with respiratory syncytial virus, were observed. The giant cells of epithelium infected with parainfluenza virus type 3 did not lose their cilia. This virus also produced changes in cells of the lamina propria and cartilage in this system with the formation of multinucleated giant cells. Immunofluorescence microscopy showed parainfluenza virus type 3 specific antigen not only in the epithelial layer but also in the lamina propria and cartilage cells. When comparable tissue was examined by electron microscopy, typical syncytial formation of epithelial cells was observed. Virus particles were observed budding off the cells in the epithelial, lamina propria and cartilage layers of the trachea.

Conclusion

In this paper I have compared the changes produced in organized, differentiated, epithelium maintained in tracheal organ culture by different infectous agents (*M. pneumoniae, B. pertussis,* respiratory syncytial virus and parainfluenza virus type 3). This simple system has demonstrated that differentiated organized epithelium responds differently to individual infectious agents. This model has been demonstrated to be useful in defining the susceptible cell type of pathogens in the organized epithelium . Only limited use of this model has been made in studying the effects of pollutants on respiratory epithelium. Adalis et al. have examined the effects of cadmium on epithelium in hamster trachael organ culture (*15*). Schiff studied the effects of nitrogen dioxide on influenza virus infection in hamster trachael organ culture (*16*). Mossman et al.

reported on the effects of carbon particles on tracheal epithelium in organ culture (*17*).

The *in vitro* tracheal organ culture model should provide a valuable screening tool to further explore the injury produced by different pollutants and to establish the lowest levels of pollutants required to produce epithelial morphological and metabolic injury. The model should also prove useful to study synergism between infectious agents and pollutants.

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