Respiratory Syncytial Virus and Human Bronchial Epithelium

Debra A. Tristram, MD; Wesley Hicks, Jr, MD; Robert Hard, PhD

Background: A suitable model for respiratory syncytial virus (RSV) infection has yet to be developed.

Objective: To describe an in vitro model of human respiratory epithelium in primary cell culture linked with a computer microscope interface that allows evaluation and imaging of living RSV-infected respiratory epithelium.

Design: A descriptive, controlled study. Human bronchial cells were obtained from surgical samples by elastase dissociation and replated on collagen gel membranes. After 7 to 10 days, cells were brought to air interface. Baseline sampling of cell fluid for cytokine production by enzyme-linked immunosorbent assay (interleukin [IL] 1β, IL-6, IL-8, and RANTES) and leukotriene C4 by radioimmunoassay was taken before treatment with RSV (n = 30) or HEp-2 (human laryngeal carcinoma cells) control (n = 25). Sampling was done at 4, 24, 72, and 120 hours thereafter. The infectious process was monitored with a microscope (Zeiss UEM, Carl Zeiss Inc, Thornwood, NY) equipped with a camera (Newvicon, Dage Corporation, Stamford, Conn). Images were either digitized using a computer (Macintosh Quadra 950, Apple Computers Inc, Cupertino, Calif) equipped with a digitizing board (Perceptive Corporation, Knoxville, Tenn) or were recorded on an SVHS videotape using a videocassette recorder (JVC, Elmwood Park, NJ).

Results: Respiratory syncytial virus induced profound effects on the ciliated cells: ciliostasis, clumping, and loss of cilia from live cells and sloughing of cells. Significant differences in the release of IL-6, IL-8, and RANTES (P<.03 for each cytokine) were noted in RSV-infected bronchial cultures by 24 hours with a peak at 72 hours. The IL-1β and leukotriene C4 were not altered by RSV infection in bronchial cells.

Conclusions: This model closely mirrors human RSV disease and affords a unique opportunity to study interepithelial cell interactions, cytokine responses from cells of different donors, and ciliary activity of live cells undergoing RSV infection.


Respiratory syncytial virus (RSV) is the single most important viral respiratory pathogen in the world. Annual RSV epidemics occur in all geographic and climatic regions. Nearly every child will have contracted at least 1 RSV infection by the age of 2 years.1,2 Immunity is incomplete after the first or second infection, and repeated infections are common.1,2 Mortality rates up to 5% have been reported in infants and children with underlying heart or lung disease, and can exceed 90% in severely immunocompromised children and adults.3-4 Despite a wealth of information derived from animal and cell culture studies, neither the treatment of RSV infection nor the development of a vaccine has been successful.3-6 Studies of infected human cell lines are poorly representative of the complex interactions that occur in the immunocompetent infected host. Organ cultures might provide a link between cell culture studies and natural or experimental human disease. However, the usefulness of animal sources for respiratory tissue is limited since animal models of RSV infection are not entirely representative of human RSV infection, particularly bronchiolitis.1-2 Overall, animal studies7,8 suggest that the target for human RSV replication may differ among species. Hence, it is also possible that the host immune response is altered depending on the pattern of RSV damage. Thus, cells of human origin are optimal to study the pathologic manifestations and pathogenesis of human RSV infection.

We have developed an in vitro model of human respiratory epithelium derived from surgical samples.9 It consists of dissociated and regrown pseudostratified respiratory epithelium containing all the cell
types present in normal respiratory epithelium, including mucus-producing cells, ciliated and nonciliated epithelial cells, and basal cells. Initially the cells lose their differentiated phenotypic characteristics, such as beating cilia. The culture medium was changed every 2 days and the epithelial surface was kept submerged. After 7 days the cells were brought to air interface (ie, fed from the bottom with 1 mL of media); no media was placed on top of the epithelial surface. This method has been successful for the consistent formation of new cilia within several days of reaching air interface. At this point our cultures closely approximate the human respiratory epithelium with the apical surface in contact with air and the basal surface attached to a supporting structure, in this case collagen. The cell cultures were monitored using an inverted microscope equipped with phase-contrast optics. Once cilia reappeared, the cells were used for experimentation.

**PREPARATION OF RSV**

Laboratory stocks of the long strain of RSV were inoculated into immortalized human respiratory cells (HEp-2) and harvested when syncytium formation was prominent (48–72 hours). Cells were frozen and thawed to maximize yield of virus, and the virus was purified by centrifugation over a sucrose gradient. Uninfected tissue culture was processed in the same fashion and purified by centrifugation to produce an adequate control antigen. HEp-2 lysate. The 43% density band was used for all purified virus experiments. Aliquots were frozen at −70°C until use so that each set of planned experiments had equivalent virus and controls available. For all described experiments, a single batch of purified RSV containing $5 \times 10^5$ plaque-forming units per milliliter was used.

**VIRUS INOCULATION**

When cells were ready for experimentation, 100 µL of the purified RSV or HEp-2 lysate was added to the surface of the cultures. Virus (or HEp-2 lysate) was adsorbed for 2 hours and then removed so that the surfaces of the cultures remained at air interface. This inoculum containing $5 \times 10^5$ plaque-forming units was calculated to produce a multiplicity of infection of 1, assuming all cells in the culture well would be targeted by RSV.

**VIRUS DETECTION**

Gentle washing of the epithelial surfaces of cell cultures was performed with 1 mL of phosphate-buffered saline.
solution at 24, 48, and 72 hours after inoculation. These samples were then inoculated into MRC-5 cells (BioWit-taker, Walkersville, Md) and processed by standard virologic methods. Respiratory syncytial virus was confirmed in infected tissue cultures by the use of fluorescent staining with a monoclonal antibody specific for RSV, as previously described. No RSV was recovered from the control cultures.

PREPARATION FOR CILIARY AND MORPHOLOGIC STUDIES

Collagen inserts containing cells were removed and mounted on a coverslip for placement in a temperature-controlled chamber. The cell media (Dulbecco minimal essential media/ Ham F-12) was replaced with a medium that is buffered with amino acids rather than bicarbonate to minimize optical interference Lebovitz L-15 (L-15, Gibco). Respiratory syncytial virus and HEp-2 lysate control were diluted in L-15 prior to addition to the tissue culture. After a 2-hour adsorption period, the virus or control media was replaced completely with new L-15. Video images were recorded throughout the infectious cycle with the use of a camera (Newvicon, Dage Corporation, Stamford, Conn). Images of the morphologic changes seen during infection were digitized using a computer (Macintosh Quadra 950, Apple Computers Inc, Cupertino, Calif) equipped with a digitizing board (Perceptics Corporation, Knoxville, Tenn). Ciliary beat frequency (CBF) was recorded on SVHS videotape using a videocassette recorder (JVC, Elmwood Park, NJ).

MEASUREMENT OF CBF

Ciliated cells were sought on the monolayer and their beat frequencies measured as previously described using a microscope (Zeiss Universal, Carl Zeiss Inc, Thornwood, NY) equipped with Nomarski differential-interference contrast optics. Briefly, a stroboscopic light source was connected to the microscope with the video recorder and computer interface to allow simultaneous measurement of CBF while continuous real time ciliary activity was filmed. Beating cilia were positioned so that their beat stroke was perpendicular to the light source. The frequency of the strobe light was varied until the CBF matched that of the strobe and the cilium appeared to freeze its motion. When beat frequencies were below 4 Hz, a stopwatch was used. Measurements of CBF were taken in all treatment groups at baseline prior to addition of virus or control media, and at 15-minute intervals during the 2-hour virus adsorption period. At each time point at least 20 measurements were taken of cilia throughout the monolayer. Means and SDs were calculated for each measurement.

CYTOKINE EVALUATION

Evaluation of the cytokine/chemokine profiles produced during RSV infection was accomplished by enzyme-linked immunosorbent assay methods. Enzyme-linked immunosorbent assay kits were purchased commercially (kits for IL-1β, IL-6 from Genzyme Corporation, Cambridge, Mass, and kits for IL-8, RANTES from R & D Systems, Minneapolis, Minn). At the onset of experimentation, all media were removed to represent a baseline sample for that well and replaced with an equal volume of culture media. Samples thereafter were taken at 2, 4, 8, 24, 72, and 120 hours after RSV or control inoculation. All samples were run in duplicate to minimize pipetting errors, and the absorbance for each sample was averaged prior to calculation of concentration.

LEUKOTRIENE C4 (LTC4) ASSAY

Leukotriene C4 was measured in the cell supernatants at baseline and at 2 and 4 hours after the addition of RSV or HEp-2 control by a radioimmunoassay (NEL, Dupont, Wilmington, Del). The assay measures LTC4 from 0.25 ng to 16 ng/mL. There is essentially no cross-reactivity with other leukotrienes except the primary metabolite of LTC4, leukotriene D4 (11%).

STATISTICAL METHODS

All statistical comparisons of data were made with a Statview 4.5 (Abacus Concepts Inc, Berkeley, Calif) package for a Macintosh computer capable of calculating parametric and nonparametric tests. Analysis of variance was used to compare the generation of cytokines and alteration in CBFs between treatment groups over time, with statistical significance considered at P<.05.

model, bovine RSV induced sloughing of the ciliated epithelium and increased concentrations of free cilia in bronchiolar lavage fluid within 24 hours of infection. In human RSV infection, loss of ciliary activity and ciliated cells may prevent airway clearing of sloughed cells and secretions, leading to mucus plugging with resultant wheezing seen during bronchiolitis of infancy. The studies herein include the effects of RSV infection on ciliary morphologic features and activity.

RESULTS

MORPHOLOGIC STUDIES

Respiratory syncytial virus treatment at 10⁻³ plaque-forming units (multiplicity of infection, 1) resulted in rapid changes in cell morphologic features. The earliest visual changes took place during viral adsorption. Abnormalities of ciliary activity and shape developed in RSV-infected cultures, but not in control cultures treated with HEp-2 lysate or L-15 media alone. Distinct cell membranes began to blur, and blebs formed on the cell surface that were released into the medium (Figure 1). Within 24 hours after RSV infection, the formation of multinucleated cells (syncytia) was clear, with fusion of adjacent cell membranes. Additional changes included spindling or rounding of cells and exfoliation of more than half of the cells. None of these changes were observed in control cells inoculated with HEp-2 lysate. Ciliary activity slowed in RSV-treated cells as early as 30 minutes after the addition of virus, and many cilia became club shaped. By 2 hours after inoculation, RSV-
treated cells had no motile cilia. By 24 hours after infection, RSV-infected tissue demonstrated loss of cilia from some cells, leaving only the basal body layer behind. Other cells had cilia, but they became fused. Still other cells had morphologically normal cilia. However, none of the cilia were motile. Finally, by 3 to 5 days following RSV infection, IL-6 release increased from a mean baseline of 259 pg/mL prior to RSV treatment to a maximum of 1400 pg/mL at 72 hours. The release of IL-6 was statistically significant at 24 hours ($P = .04$) and at 72 hours ($P = .02$) when comparing RSV-infected cells with their own baseline production. By analysis of variance for repeated measurements, RSV-treated cells released more IL-6 than control cells ($P = .03$). The HEp-2 control wells had slightly higher mean baseline release of IL-6 (325 pg/mL) but this was not statistically different compared with IL-6 release from the RSV-treated wells prior to RSV treatment. At 4 hours and 24 hours after HEp-2 lysate treatment, IL-6 release increased but not significantly ($P = .11$). By 72 hours, IL-6 release was essentially at baseline values in the control wells.

RANTES release during RSV or HEp-2 lysate treatment is shown in Figure 2. Respiratory syncytial virus–treated cells released significant RANTES as early as 4 hours after RSV infection but the release was earlier, during the first 24 hours, and decreased rapidly ($P = .004$). Cells from the remaining 5 individuals had moderate baseline IL-8 levels with increased release above baseline production in response to RSV infection.

RANTES release during RSV or HEp-2 lysate treatment is shown in Figure 2. Respiratory syncytial virus–treated cells released significant RANTES as early as 4 hours after RSV infection of cultured human bronchial cells ($P = .01$). At 24 hours, the mean release of RANTES...
was still significantly different from baseline and from control cells \((P = .03)\). Mean peak RANTES release of 780 pg/mL was reached by day 3 in RSV-infected cells only \((P = .03; \text{Figure 3})\). Control cells did not release more than 112 pg/mL of RANTES at 24 hours when exposed to HEp-2 lysate, and fell back to 0 after 24 hours.

**LEUKOTRIENE C4**

The quantity of LTC4 was measured in the cell supernatants at baseline, 2 and 4 hours after the addition of RSV, or in Hep-2 control by radioimmunooassay. We were unable to detect LTC4 in either the RSV-infected or control cultures at these early time points when ciliostasis occurred in RSV-infected cells.

**CILIARY ACTIVITY DURING RSV INFECTION**

Ciliary beat frequency was monitored prior to the addition of RSV or control media and every 15 minutes during the 2-hour virus adsorption period (Figure 3). Within 30 minutes of the addition of live RSV, changes in CBF were noted. By 60 minutes, decreased beat frequency with discoordinated activity was noted in RSV treatment groups only. All cilia ceased beating in the RSV-treated group by 2 hours. Control cultures with HEp-2 lysate or L-15 alone continued to beat within the normal frequency range throughout the observation period.

The most unexpected finding was the temporary restoration of CBF to normal levels when fresh L-15 was added to the RSV-infected cells at the end of the 2-hour period for virus adsorption. Addition of fresh media to the HEp-2 lysate or L-15 media control cultures had no effect on CBF. Evaluation of RSV-infected cultures at 24 hours after inoculation revealed no active cilia for measurement of CBF, while the cilia in all uninfected cell cultures were beating normally.

**COMMENT**

To our knowledge, this is the first model for RSV infection of its type to be described in the literature. We used mixed cell cultures of human respiratory origin that differ from previous human respiratory tissue explants in...
several important ways. They are not isolated cells brushed from the respiratory tract, hence removed from the normal milieu of the respiratory mucosa. Such cells have limited viability and are not capable of regeneration or long-term experimentation. In our model, respiratory tissue is dissociated to single cells for removal of all contaminating connective tissue and other components of tissue that are not epithelial in origin, unlike older organ culture models or respiratory epithelial explants. Our model of ciliated human epithelial cultures can be infected with RSV and demonstrates cytopathic effects characteristic of human RSV infection. Our RSV-infected cells produce cytokines similar to those seen in natural RSV infection and in standard tissue culture models. These results validate our hypothesis that our model of RSV infection mirrors that of natural and experimental human RSV infection. We have the unique opportunity to film the infectious process over time and record the development of pathologic features, particularly alterations in ciliary function, without destruction of living cells through fixation techniques.

Airway epithelial cells are the primary initiators of pulmonary host defense mechanisms by their ability to synthesize and secrete inflammatory mediators on injury or infection. Elevated levels of IL-1β in nasopharyngeal secretions and increased messenger RNA transcripts specific for IL-1β in nasal biopsy tissue were found in children with acute viral upper respiratory tract infection. We also expected to find the production of IL-1β to be altered in our RSV-infected cells. Our bronchial tissue cultures produced measurable quantities of IL-1β, but did not increase in response to RSV infection. Perhaps the cells that normally produce IL-1β were damaged during RSV infection, and incapable of increased synthesis and release of IL-1β. The IL-1β messenger RNA transcripts in nasal biopsy tissue may reflect early epithelial repair due to modulation of the extracellular matrix and not the result of infection per se. Interleukin 1β has been implicated in the upregulation of keratinocyte growth factor in fibroblast cells, and therefore may participate in the process of repair as well as inflammation. Although our model is capable of self-repair from focal chemical injury, ongoing viral damage may preclude repair of our small epithelial cultures. Another possible explanation may lie with the epithelium itself. While nasal epithelium may produce IL-1β in response to RSV infection (or repair), lower respiratory tract epithelium may not. Fieldler et al found that neither A549, BEAS 2B, nor H441 cells demonstrated increased release of IL-1β in response to RSV infection; this correlates with our findings in bronchial epithelial cultures.

Locally produced cytokines, including IL-6 and IL-8, have been detected in the nasal lavages of children with viral infection of the upper respiratory tract. Interleukin 6 may augment antibody synthesis from B cells and enhance differentiation of cytotoxic lymphocytes from T cells. Interleukin 8 and RANTES have a variety of functions, but most relevant for the study of RSV are their chemotactic activities for neutrophils and eosinophils, respectively. Since cytotoxic lymphocytes participate in the eradication of RSV, local production of IL-6 by infected epithelium may stimulate mucosal lymphocytes prior to the arrival of “secondary” inflammatory cells recruited from the systemic circulation. A549 cells upregulate IL-8 production and release in response to RSV infection alone and in conjunction with soluble factors released during coculture with both peripheral blood lymphocytes and neutrophils. RANTES is also upregulated during RSV infection of standard tissue culture and in primary cultures of nasal epithelium. Our RSV-infected cells produced elevated levels of IL-6, IL-8, and RANTES only with replicating virus. There was a considerable variation in measurable IL-8 between subjects, but consistent results within cells from the same donor. One individual produced 10 times the IL-8 at baseline compared with the baseline production from the other subjects; IL-8 release for these cells still increased significantly in response to RSV infection. Thus, individual differences in responsiveness to RSV by altered IL-8 release (and probably other factors as well) may explain degrees of severity encountered in RSV disease of humans.

Ciliary activity is a critical arm of the mucociliary transport system, but has not been fully explored during human RSV infection. Henderson and colleagues observed altered ciliary activity and loss of cilia in their human fetal tracheal rings but did not quantitate their findings. Respiratory syncytial virus produced ciliostasis early in the infectious cycle in our study, well before viral replication took place. Mechanisms involved in ciliostasis are poorly understood but could be mediated by ion alterations during viral attachment and replication, direct damage to the ciliary apparatus, or damage through the production of a toxic substance, such as LTC4, by the infected cell or adjacent cells. Hisamatsu et al demonstrated that LTC4 inhibited ciliary activity in a time- and dose-dependent manner in nasal epithelial cells. The changes in CBF induced by 10−6 to 10−9 mol of LTC4 were not significantly different from baseline activity until at least 4 hours after exposure; complete ciliostasis took much longer. Ciliostasis in our model is rapidly induced, suggesting that either a higher concentration than 10−6 mol of LTC4 is released by infected cells, or alterations in ion concentrations are involved. We were unable to detect any LTC4 in our RSV-infected or control cultures at the time ciliostasis occurred, suggesting that the level of LTC4, if present, was lower than the limits of detection by the assay (<0.25 ng/mL). The RSV-infected cells were clearly not dead, yet fresh media were capable of temporarily restoring ciliary activity. In RSV-infected wells, all ciliated cells were involved. However, ciliostasis may not be so widespread in vivo, since it is unlikely that one would inhale or replicate sufficient RSV to infect every target cell in the respiratory tract. In vivo, localized disruption in the mucociliary transport might cause disorganized mucus and cellular debris removal, thus contributing to the clinical features of air trapping and wheezing seen during RSV bronchiolitis of infancy. We intend to pursue these speculations of ciliostasis in future studies.


©1998 American Medical Association. All rights reserved.
Our model of differentiated, ciliated human respiratory epithelium provides a unique in vitro model consisting of the type of cells present in the normal human respiratory tract: basal, secretory, and ciliated. Respiratory syncytial virus infection in our model closely mirrors human disease and affords an opportunity to study interepithelial cell interactions and ciliary activity of live cells. We can evaluate cytokine production in response to RSV infection from cells of different donors as opposed to cells from cell lines that derive from only a single donor. Finally, our study offers a distinct advantage of assessing functional and morphologic changes that occur during active RSV infection including ciliary dysfunction, which is an important feature in the pathogenesis of clinical bronchiolitis.

Accepted for publication April 14, 1998.

This work was supported by a grant from the Women and Children's Health Research Foundation of Children's Hospital of Buffalo, Buffalo, NY (Dr Tristram).


We thank Leon Hall and Ezzat Assian, MS, for their expert technical assistance and Deborah Reszak for her secretarial support.

Corresponding author: Debra Tristram, MD, Division of Infectious Disease, Children's Hospital, 219 Bryant St, Buffalo, NY 14222 (e-mail: dtristra@acsu.buffalo.edu).

REFERENCES

12. Makela MJ, Ruuskanen O, Ogra PL. Treatment of respiratory syncytial virus infection with ribavirin during active RSV infection including ciliary dysfunction, which is an important feature in the pathogenesis of clinical bronchiolitis.

©1998 American Medical Association. All rights reserved.