

## Original Research

# Acute and Chronic Airway Disease After Human Respiratory Syncytial Virus Infection in Cotton Rats (*Sigmodon hispidus*)

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Infection with respiratory syncytial virus (RSV) generally presents as a mild, upper airway disease in human patients but may cause severe lower airway disease in the very young and very old. Progress toward understanding the mechanisms of RSV pathogenesis has been hampered by a lack of relevant rodent models. Mice, the species most commonly used in RSV research, are resistant to upper respiratory infection and do not recapitulate the pattern of virus spread in the human host. To address the need for better rodent models of RSV infection, we have characterized the acute and chronic pathology of RSV infection of a relatively permissive host, cotton rats (*Sigmodon hispidus*). We demonstrate that virus delivered to the upper airway results in widespread RSV replication in the ciliated respiratory epithelial cells of the nasal cavity and, to a lesser extent, of the lung. Although acute inflammation is relatively mild and rapidly eliminated after viral clearance, chronic, eosinophilic lung pathology persists. These data support the use of cotton rats as a robust rodent model of human RSV disease, including the association between RSV pneumonia and subsequent development of allergic asthma.

**Abbreviation:** BAL, bronchoalveolar lavage; RSV, human respiratory syncytial virus.

Human respiratory syncytial virus (RSV) is the primary cause of lower airway disease in infants and children worldwide.<sup>39</sup> Although generally limited to the upper airway, RSV infection can also manifest as bronchiolitis and pneumonia in young children and the elderly and has been implicated as a major cause of middle ear infections (otitis media).<sup>3,55</sup> In addition to the problems associated with acute illness, children who experience severe RSV disease in infancy are at increased risk for development of asthma and recurrent wheezing later in childhood.<sup>35,53,56</sup>

RSV is ubiquitous worldwide, with most children infected in infancy, and essentially all children are infected by 3 y of age.<sup>20</sup> Although reinfection throughout life has been documented, the true incidence of upper airway infection is difficult to quantify, given that treatment for such infections typically is not sought. In comparison, lower airway infection is more likely to come to medical attention, and it is currently estimated that at least 33.8 million episodes of RSV-induced acute pulmonary infection in children younger than 5 y occur yearly and that as many as 200,000 of those episodes are fatal.<sup>40</sup> Although supportive treatment for severe RSV disease is highly effective in infants, access to such treatment is generally only available in industrialized countries. More than 90% of fatal RSV cases occur in the developing world.<sup>40</sup>

Mice have been used extensively to study RSV infection, yet there are key limitations of the mouse model for the study of human RSV disease and immunity. The most important of these is the poor permissiveness of the mouse for human RSV. RSV replicates to a limited extent in the mouse lung, and large viral loads delivered in a relatively large volume are generally used with this model. Even with intranasal inoculation, primarily lower, and not upper, airway infection is achieved in WT mice. This scenario is unlike the human disease, in which the upper airway is the primary target of RSV replication.<sup>24,25</sup> Mice are essentially resistant to upper airway infection, with little to no virus detected in nasal washes evaluated by plaque assay, and only rare RSV-antigen-positive cells are detected by immunohistochemistry.<sup>13,18,21</sup> In addition to this altered route of entry, the pattern of lung infection is markedly divergent between humans and mice. In humans, RSV primarily infects ciliated bronchiolar epithelial cells and, to a much lesser extent, alveolar cells.<sup>32</sup> In contrast, bronchiolar epithelial cells are infected only rarely in the mouse lung; instead RSV targets the pneumocytes.<sup>37</sup>

Unlike mice, cotton rats are susceptible to RSV infection of the upper airway, and the pattern of lower airway infection mirrors that seen in human patients. The cotton rat was established as a model of RSV infection more than 3 decades ago and has since emerged as the preferred rodent model in which to evaluate RSV therapeutics and vaccine candidates.<sup>19,23,33,41,49,57,60</sup> In addition, as the availability of species-specific reagents has improved, cotton rats have become an increasingly useful model in which to study RSV pathogenesis.<sup>6</sup> However, whereas the susceptibility of cotton rats to RSV infection has been established firmly, the

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pathology of RSV infection in this species has not yet been extensively characterized.

Asthma clearly is a multifactorial disease, dependent on both genetic and environmental factors (see references 28 and 30 for recent reviews), and many studies have pointed to a role for respiratory virus infection in the induction of asthma. In one study,<sup>17</sup> the combination of atopy and the presence of virus in nasal secretions synergistically increased the odds ratio for wheezing in children 25-fold, and another study<sup>29</sup> showed that repeated rhinovirus infections in the first 3 y of life increased by 26-fold the risk of developing asthma by the age of 6 y. This relationship is obviously a complex one, influenced both by the nature and the timing of the viral infection. Nonetheless, as many as 80% of acute asthma exacerbations in children and approximately 50% in adults are associated with viral infection.<sup>33,42</sup> The majority of these infections are attributed to rhinoviruses, but other respiratory viruses, including influenza virus, RSV, and coronaviruses, can provoke these attacks.<sup>33,42</sup> Beyond the exacerbation of established asthma, evidence that severe RSV disease in infancy is correlated with development of asthma and recurrent wheezing in later childhood is mounting. An association between lower airway RSV infection and subsequent development of recurrent wheezing and asthma was demonstrated more than 30 y ago,<sup>16</sup> and several recent prospective studies have strengthened this correlation.<sup>27,53</sup>

In the current study, we expand upon existing knowledge of the cotton rat model of RSV infection by characterizing the spread of virus after droplet inhalation and the early and late inflammatory response to viral challenge. We confirm the observation that cotton rats are relatively permissive in regard to RSV infection as compared with mice, and we demonstrate that the infection of cotton rats, like that in the human host, is primarily an upper airway phenomenon. In addition, we show that primary RSV infection of the cotton rat lung, even at the low levels in this study, induces chronic changes consistent with those in cases of human allergic asthma. This finding is of interest because of the substantial literature associating early, severe respiratory infection with the development of asthma later in life. The studies of RSV infection in cotton rats that we describe here suggest that this species may be predisposed to atopy and that, long after virus clearance, changes associated with allergic inflammation persist in human hosts. Therefore, in addition to the usefulness of cotton rats for testing RSV treatments and vaccine candidates, we suggest that this species may serve as a model system for determining the involvement of virus infection in the development of allergic asthma.

## Materials and Methods

**Cotton rats.** Female cotton rats (*Sigmodon hispidus*; age, 6 to 10 wk; Sigmovir Biosystems, Bethesda, MD) were used in this study. Cotton rats were housed in groups of 4 in a barrier facility and received a commercial pelleted rat chow and water free choice. On the basis of physical examination by a licensed veterinarian, all cotton rats were considered healthy on arrival at our facility. Testing for specific pathogens was not performed, because naturally occurring infectious diseases of cotton rats are not well characterized. The cotton rats were acclimated for 7 to 10 d prior to viral challenge. All procedures used here were conducted humanely. Animal housing and experimental procedures were approved by the IACUC at the New York University School of Medicine and

Rutgers New Jersey Medical School. Cohorts of 4 to 5 animals were used for each experimental point.

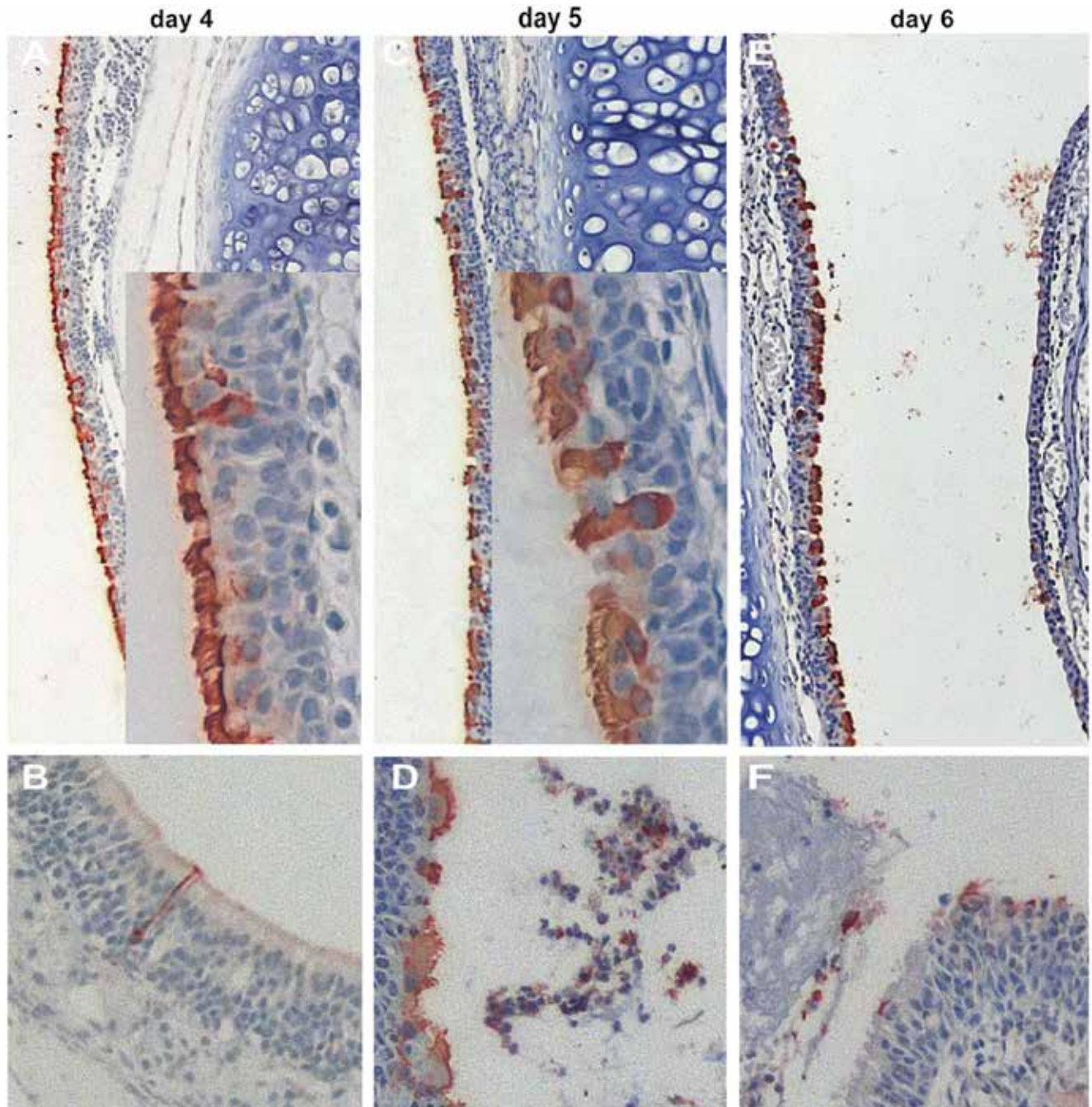
**Virus and viral infection.** Human RSV strain A2, originally obtained from R Chanock at the NIH, was passaged on murine STAT1<sup>-/-</sup> fibroblast monolayers. For preparation of virus stock, STAT1<sup>-/-</sup> fibroblasts were infected at a multiplicity of infection of approximately 0.02 and incubated in DMEM with 5% fetal calf serum for 36 to 48 h until nearly all cells were involved in syncytia. Medium was collected and centrifuged at 1000 × g for 5 min, then pelleted cells were resuspended in E3E (3% ethanol, 0.025 M glucose, 0.15 M NaCl, 0.00533 M KCl, 0.001 M Na<sub>2</sub>EDTA, 0.00916 M Na<sub>2</sub>HPO<sub>4</sub>, 0.025 M HEPES pH 7.6, 0.0015% phenol red), 1 mL per 60 cm<sup>2</sup> cells. This preparation was centrifuged at 3000 × g for 5 min. The supernatant was then centrifuged at 20,000 × g for 2 h and the resulting pellet resuspended in 0.1 M NaCl, 0.02M HEPES pH7.6, 0.005M MgCl<sub>2</sub>. After an additional centrifugation at 3000 × g for 5 min, the resulting supernatant was used as virus stock. In experiments using UV-inactivated virus, the virus was inactivated by exposure to a 30-W germicidal UV bulb for 2 min at a distance of 60 cm from the source.

While under isofluorane anesthesia, cotton rats were intranasally challenged with 1 × 10<sup>6</sup> pfu RSV (or were mock challenged with sterile saline) in a 50-μL total volume divided equally between nares. The viral inoculum was delivered slowly, over approximately 15 s, in a small volume to restrict initial virus infection to the upper airway; 8 cotton rats were used for each time point. Saline-challenged controls were examined at a single time point only, that is, 1 d after saline instillation. Infected cotton rats were examined on days 2, 4, 5, 6, 7, 10 and 28 after inoculation. At each time point, nasal wash and bronchoalveolar lavage (BAL) fluids were collected from 4 cotton rats; lung (right lung for plaque assay and left lung for histology and immunohistochemistry) and nasal cavities (for histology and immunohistochemistry) were collected from the remaining 4 cotton rats in the group. Thus, each sample or tissue was used for only one purpose (for example, lung histology was not performed on lungs that had been lavaged with saline). After inoculation with live or UV-inactivated virus, cohorts of 5 cotton rats were euthanized, with collection of BAL fluids and lung tissue, at day 5 after treatment.

**Viral plaque assay.** Cotton rats were euthanized by CO<sub>2</sub> asphyxiation at multiple time points until 28 d after virus challenge. Nasal wash fluids and lungs were collected immediately after euthanasia and stored at -80 °C until use. Viral plaque assay was performed on murine STAT1<sup>-/-</sup> fibroblast monolayers as previously described.<sup>18</sup>

**BAL analysis.** Immediately after CO<sub>2</sub> asphyxiation of cotton rats, BAL fluids were collected by washing the lung with 2 mL of sterile saline. Cell differentials were determined on Wright-Geimsa-stained preparations (CytoSpin, Thermo Scientific, Waltham, MA).

**Histology and immunohistochemistry.** Tissue specimens were collected immediately after CO<sub>2</sub> asphyxiation. Nasal cavities were fixed in neutral buffered formalin then decalcified with 0.35 M EDTA in 0.1 M Tris (pH 6.95). The right lung and decalcified nasal cavities were processed routinely, paraffin-embedded and sectioned at 5 μm. Tissues sections were stained with hematoxylin and eosin or were left unstained for immunohistochemistry. For immunohistochemistry, tissue sections were incubated with goat polyclonal RSV antiserum (Biodesign, Saco, ME) diluted 1:500 followed by incubation with biotinylated rabbit anti-goat antibody (ScyTek, Logan, UT), streptavidin linked to horseradish



**Figure 1.** Distribution and extent of RSV infection of the upper airways of cotton rats. Nasal cavity tissues were collected from cotton rats at multiple early time points after RSV infection and examined for RSV antigens by immunohistochemistry. Widespread infection of ciliated respiratory epithelial cells was detected in 100% of cotton rats (A) 4 d, (C) 5 d, and (E) 6 d after RSV infection, with peak infection observed at the 5-d time point. Infection was not limited to ciliated respiratory epithelial cells but also was detected in neuronal cells of the olfactory epithelium (B, 4 d after RSV infection). On days 5 and 6, mucus with sloughed, RSV-antigen-positive, epithelial cells was present in upper airway lumina (D and F). Images were captured at magnifications of 100× (A, C, E), 200× (B, D, F), and 400× (insets).

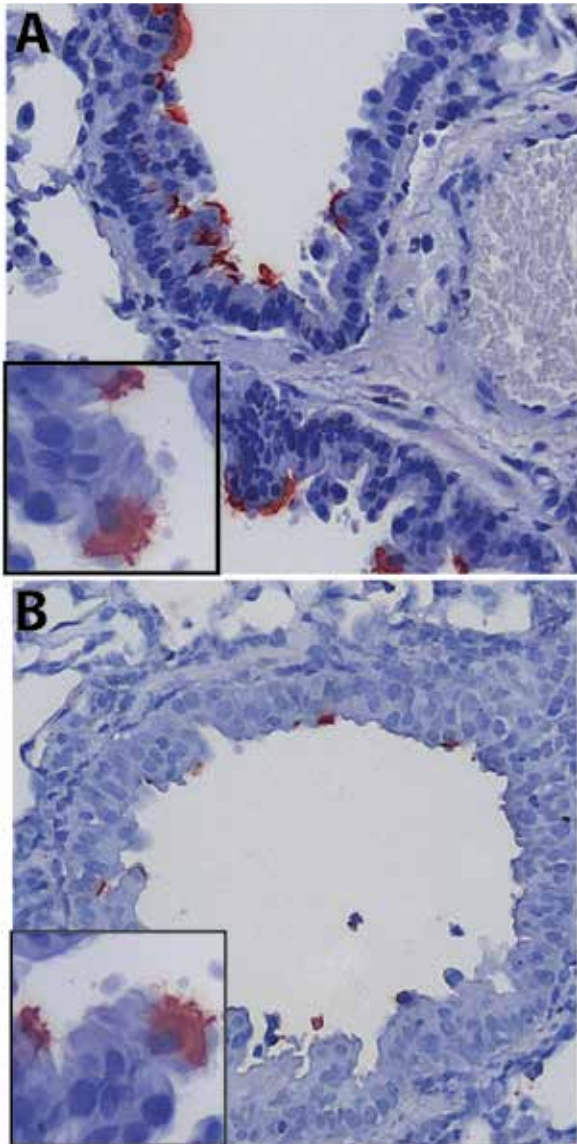
peroxidase, and 3-amino-9-ethylcarbazole chromagen (Scytek). Labeled tissue sections were counterstained with hematoxylin. Parental consent was obtained for the use of autopsy material for research.

**Statistical analysis.** SigmaPlot 12.0 (Systat Software, San Jose, CA) was used to perform Student *t* tests where appropriate. A *P* value less than 0.05 was considered statistically significant.

## Results

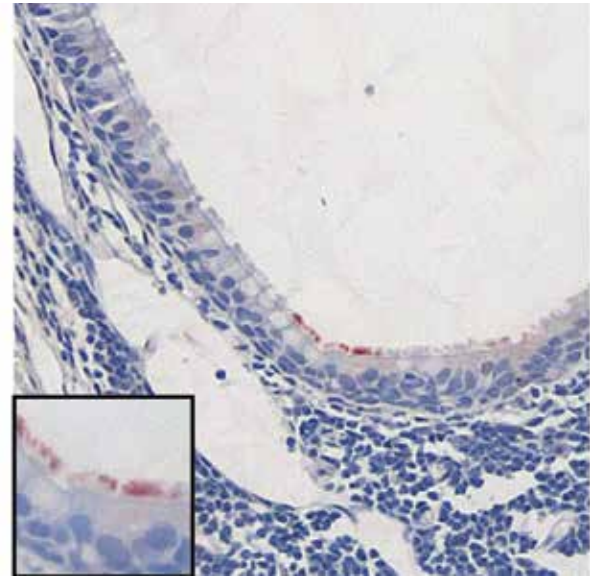
**Intranasal RSV inoculation causes diffuse upper airway and focal lower airway infection in cotton rats.** Although the cotton rat is considered the ‘gold standard’ for preclinical evaluation of RSV therapeutics and vaccine candidates, the pathology of RSV infection in this species has not been extensively characterized. To address this gap, we performed intranasal RSV infections of cotton





**Figure 2.** Distribution and extent of acute RSV infection of the cotton rat lower airway. Lung tissue was collected from cotton rats at multiple time points early after RSV infection and examined for RSV antigens by using immunohistochemistry. Sporadic infection of ciliated respiratory epithelial cells lining bronchioles was detected in the lungs of 100%, 75%, and 25% of rats at 2 d, (A) 4 d, and (B) 6 d after RSV infection, respectively. Lung involvement, always focal, decreased markedly by 6 d after infection with detection of only rare, weakly antigen-positive cells, with antigen restricted to the cilia. RSV antigen was not detected in lung collected after the 6-d time point (not shown). Images were captured at a magnification of 200× or 400× (insets).

rats by using a small-volume viral inoculum, delivered slowly, to restrict initial infection to the nasopharynx. We then tracked viral replication and associated pathology during the acute infection and at a late time point (28 d after infection), when active virus infection had resolved. Virus replication was assayed in the nasal cavity and lung tissues via immunohistochemistry and plaque assay. As expected, plaque assay results were consistent with those published previously,<sup>46</sup> with peak lung titers on the order of  $10^4$  to  $10^5$  pfu per gram of tissue and nasal wash titers of  $10^3$ – $10^4$  pfu/mL. Immunohistochemistry for RSV antigens showed diffuse viral

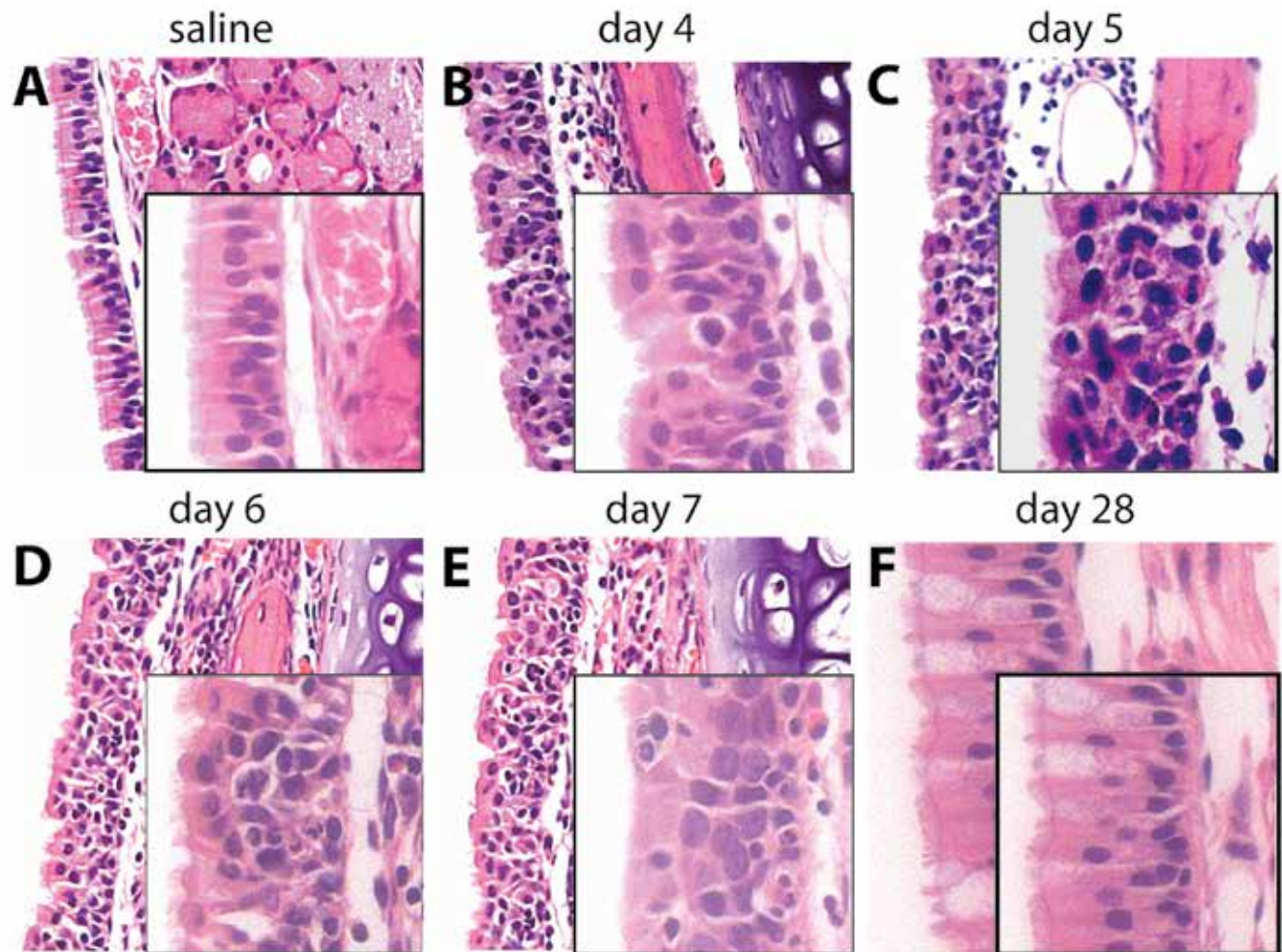


**Figure 3.** Persistence of RSV infection in the upper airway of cotton rats. Nasal cavity tissues were collected from cotton rats 28 d after RSV infection and examined for RSV antigens by using immunohistochemistry. Rare antigen-positive ciliated respiratory epithelial cells were detected. Epithelium shown here overlies the nasal associated lymphoid tissue (NALT). Images were captured at a magnification of 200× or 400× (inset).

replication in the nasal cavity, which peaked on day 5 (Figure 1), but only scattered foci of viral infection were seen in the lung (Figure 2). By day 4 after infection, the peak of viral replication in the lung, RSV antigen was visualized in the lungs of 75% of rats, dropping to 25% at day 6, when extensive upper airway infection was still present in 100% of rats. RSV antigen was not detected in the lung beyond day 6 (data not shown). No clinical signs of illness were apparent at any time point. These findings are in agreement with previous work.<sup>46</sup>

Like in human patients but unlike in mice, the ciliated respiratory epithelial cells, which line the nasal cavity, were the primary target for RSV infection in cotton rats.<sup>26,32</sup> However, in addition to these cells, we noted RSV infection of the olfactory epithelium of the caudal nasal cavity (Figure 1 B). The olfactory epithelium, which lines the rostral portion of the nasal cavity, comprises bipolar olfactory neurons surrounded by supporting sustentacular cells and the excretory ducts from submucosal mucus glands. Viral antigen was present in the ductal epithelium and neuronal cell bodies. We previously noted RSV infection of the olfactory epithelium in both the mouse and chinchilla models;<sup>18</sup> this pattern has not been observed in human patients, but examination of this tissue is not standard medical practice. As acute infection progressed through day 6, the upper airway contained abundant mucus with sloughed epithelial cells, many of which were positive for RSV antigens (Figure 1 D and F). Copious mucus production is a feature of RSV infection in human hosts.<sup>9</sup>

**RSV infection persists in the upper airway of cotton rats.** One of the remaining uncertainties in RSV epidemiology is the basis for seasonal outbreaks. RSV infection occurs only in the winter months in temperate climates, yet no animal reservoir, which could sustain the virus between human outbreaks, has been identified. Although not demonstrated convincingly, low-levels of virus are suspected to be harbored in the upper respiratory tract of some subjects throughout the year. During seasonal outbreaks,



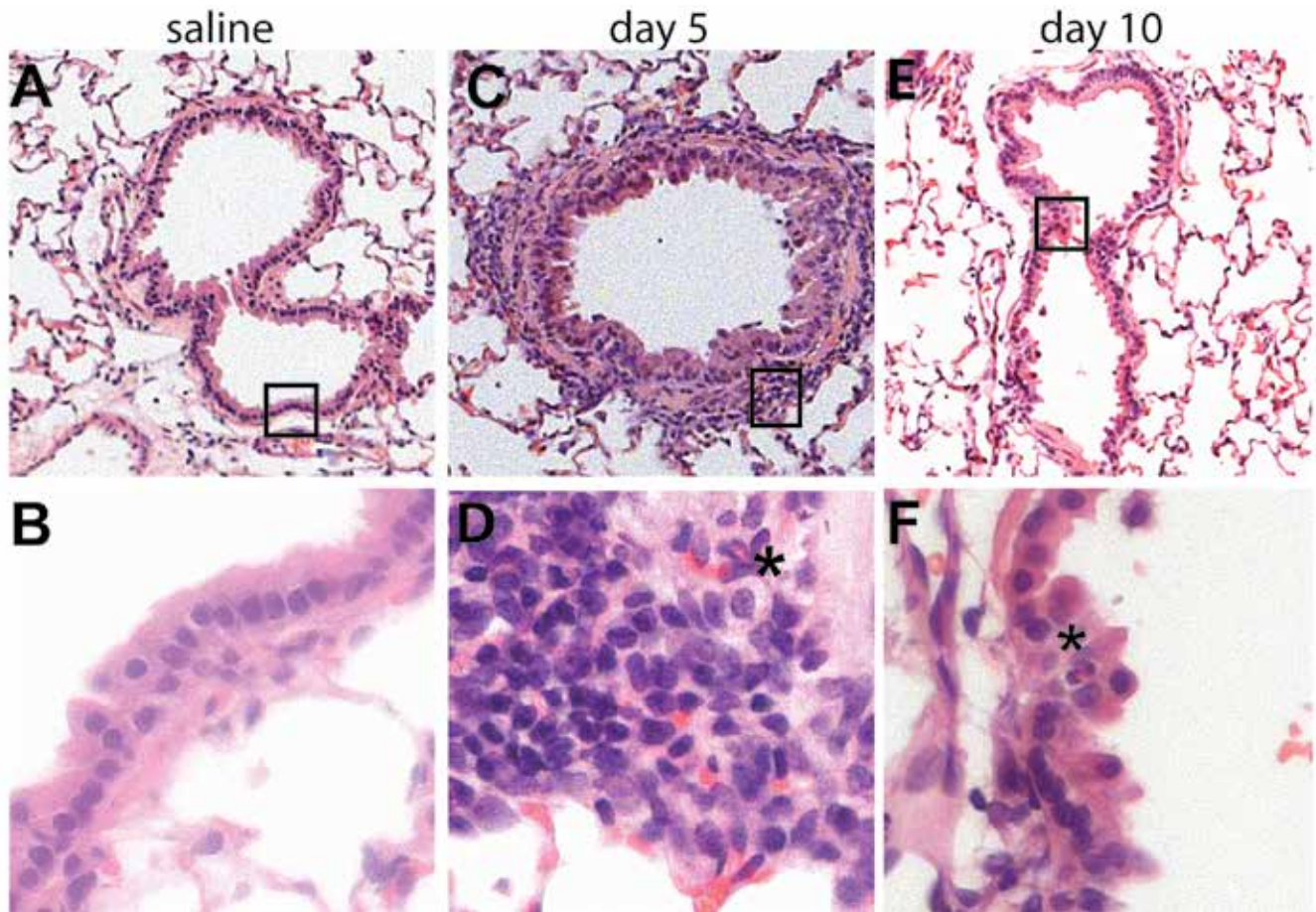
**Figure 4.** Histopathology of acute and chronic RSV infection of the upper airway in cotton rats. Histology was performed on tissues from the nasal cavity collected until 28 d after viral challenge. The surfaces of the nasal septum and turbinates in the anterior portion of the nasal cavity are covered primarily by columnar or pseudocolumnar respiratory epithelium, and sections from that area are shown here. (A) Naïve upper respiratory mucosa is characterized by an orderly arrangement of ciliated respiratory epithelial cells with small numbers of more granular-appearing, mucus-producing, goblet cells. RSV infection resulted in acute inflammation most severe at the 5-d time point, with resolution beginning by day 6. (B) A mixed inflammatory infiltrate in the epithelial layer and the submucosa was present in nasal mucosa collected 4 d after infection. (C) Increasingly severe inflammation was present 5 d after RSV infection. (D) By 6 d after RSV infection, inflammation persisted, but early evidence of epithelial regeneration emerged, characterized by basal cell proliferation. (E) Lingering histopathologic evidence of inflammation, including intraepithelial abscesses and loss of cilia, persisted at the 7-d time point. (F) Finally, regeneration of nasal cavity mucosa and resolution of inflammation was essentially complete by 28 d after RSV infection, with only goblet cell metaplasia remaining. Images were captured at a magnification of 200× or 400× (insets).

for reasons unknown, virus replication resumes, virus particles are shed, and infection of the human population is maintained.<sup>52,54</sup> In support of this hypothesis, we previously demonstrated that viral antigen can be detected in the upper airway of chinchillas as long as 14 d after RSV infection, when productive infection is no longer detectable by plaque assay.<sup>22</sup> We sought to determine whether the upper airway of cotton rats is similarly permissive to persistent RSV infection. To this end, we collected nasal cavities 28 d after RSV infection and performed immunohistochemistry for RSV antigen. Low levels of RSV antigen were present in the upper airways of 100% of rats at this late time point (Figure 3). This result was in contrast to that for the lower airway, which was negative for virus by 6 d after infection (data not shown).

**RSV infection of the upper airway of cotton rats triggers a mixed inflammatory infiltrate followed by rapid resolution.** The upper

airway of cotton rats, as compared with mice, has long been recognized as permissive for RSV replication. Nonetheless, most studies using this model have focused on lung, rather than nasal, pathology.<sup>8,14,34,59</sup> Because RSV is primarily an upper airway infection in humans, we wanted to characterize disease that followed intranasal delivery of a small volume of inoculum rather than a volume sufficient to infect the nose and lung simultaneously, as had been done previously.<sup>46</sup> Using the small-volume approach, we observed that the diffuse virus spread was accompanied by a mixed inflammatory response that peaked at day 5 after infection but was essentially resolved by the 28-d time point despite evidence of viral persistence. The infiltrate was composed of neutrophils, eosinophils, macrophages, and lymphocytes and involved the epithelium and underlying submucosa (Figure 4 A through C). Mucosal injury was evident by day 5, with regen-





**Figure 5.** Histopathology of acute RSV infection of the lower airway in cotton rats. Lungs collected at 5 and 10 d after virus challenge were processed after fixation; and hematoxylin- and eosin-stained sections were examined. (A–C) Naïve lung is characterized by thin alveolar septate and bronchioles lined by an orderly arrangement of ciliated respiratory epithelial cells surrounded by a thin layer of smooth muscle. (D–F) By 5 d after infection, there were multiple foci of bronchiolitis, with mucosal and peribronchial infiltrates consisting primarily of lymphocytes and histiocytes, with rare eosinophils (\*). Some areas showed involvement of adjacent alveolar septae. Mucosal changes included epithelial disorganization and hyperplasia. (F) By 10 d after infection, inflammation had largely resolved, with only rare eosinophils present within the bronchiolar epithelium (\*). Mucosal repair was ongoing at the 10-d time point, characterized by epithelial hypertrophy and squamous metaplasia. Images were captured at a magnification of 40× (top panels), 100× (middle panels), or 400× (bottom panels). Areas shown at higher magnification in the lower panels are highlighted by boxes in upper-panel images.

erative changes, marked by basal cell proliferation, observed as early as 6 d after RSV infection (Figure 4 D). Resolution of nasal mucosal inflammation was essentially complete by 28 d after RSV infection, in that only rare inflammatory cells remained, but goblet cell metaplasia persisted (Figure 4 F).

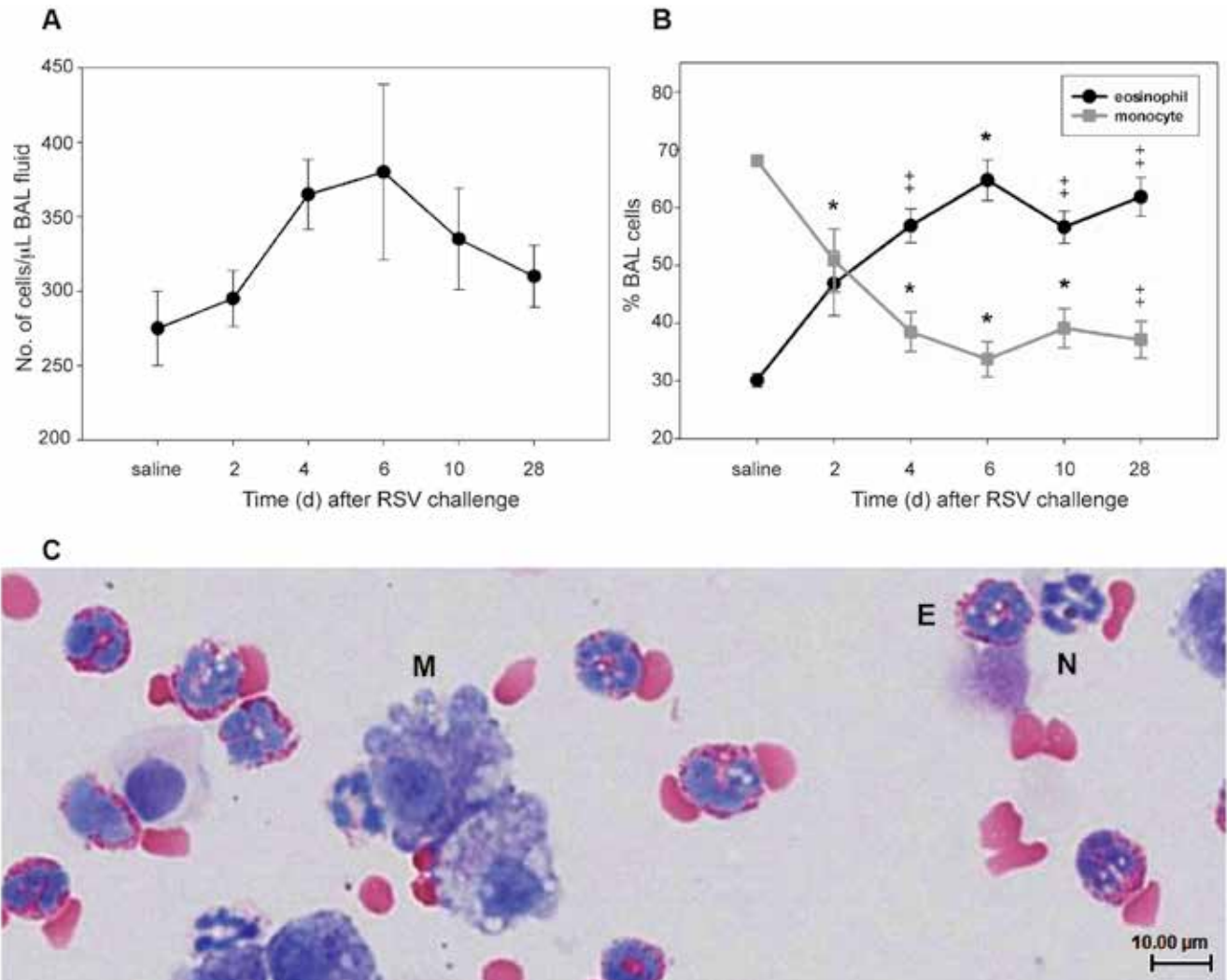
**RSV infection of the lung of cotton rats is characterized by rapidly resolving bronchiolitis and persistent eosinophilia.** Given the clinical significance of RSV pneumonia, most rodent studies have focused on the effects of RSV infection in lung. Nonetheless, because of our interest in vaccine design and the necessity of determining whether any aspect of pathology after immunization challenge is enhanced relative to infection alone, we sought a more complete picture of the development and resolution of lower airway infection and inflammation in the lung of RSV-infected cotton rats.

We noted moderate, multifocal, peribronchiolar inflammation, with occasional involvement of the alveolar septae, which was maximal 5 d after RSV inoculation (Figure 5 C and D). Inflamma-

tion was characterized by a primarily lymphocytic infiltrate that also contained macrophages and eosinophils. Alveolar septae adjacent to foci of bronchiolitis were expanded by this infiltrate, and a small number of inflammatory cells were present within alveolar spaces. Eosinophils were present within the epithelial layer.

Coincident with inflammatory influx were bronchial and bronchiolar epithelial changes. Unlike the extensive epithelial necrosis seen in influenza or adenoviral infections,<sup>26</sup> dropout of individual epithelial cells occurred after RSV infection. The mucosal layer remained intact, but showed acute changes in cell morphology, for example, loss of cilia and a cuboidal rather than columnar appearance. This pattern was followed by regenerative changes, with hypertrophy of basal cells. The acute inflammatory response resolved quickly, and by day 10 after infection, only rare eosinophils remained (Figure 5 E and F).

For a more quantitative assessment of the inflammatory process. BAL fluids were examined at 2, 4, 6, 10, and 28 d after inoculation. We noted a mild increase in BAL fluid cellularity (Figure 6 A)



**Figure 6.** Cytology of RSV infection of the lower airway in cotton rats. BAL fluid was collected from cotton rats until 28 d after RSV infection, and cytopsin preparations were examined after fixation. (A) RSV infection induced a mild, not statistically significant, increase in total number of BAL cells that was most pronounced at the 6 d time point. (B) Differential cell counts revealed a marked increase in the percentage of eosinophils as early as 2 d after RSV infection. The increase ( $P < 0.001$ ) in eosinophil percentage was accompanied by a corresponding decrease in monocyte percentage and persisted to 28 d. The increase in eosinophil percentage is supported by (C) BAL cytology (magnification, 400 $\times$ ), where eosinophils (E) were differentiated from neutrophils (N) by the presence of abundant brightly eosinophilic cytoplasmic granules. Monocytes (M), the second-most predominant cell type observed, are large cells characterized by abundant basophilic granular to vacuolated cytoplasm and frequently ruffled cell borders. \*,  $P < 0.05$ ; †  $P < 0.001$ ; data points represent the mean (error bars, SEM).

that peaked on day 6 and returned to baseline by the 28-d time point. However, despite only modest changes in overall cell numbers, BAL fluid cytology revealed a marked shift in cellular composition in response to RSV infection (Figure 6 B). At baseline, after saline challenge, BAL fluid was composed predominantly of macrophages. This finding was not unexpected, given that the BAL fluid from healthy animals of many species, including humans, is composed predominantly of this cell type. What was unexpected—and previously unreported—was the presence of eosinophils, averaging 30% of the total cell number, in unchallenged cotton rats. After RSV infection, the number of eosinophils doubled, becoming the predominant cell type present in the airways throughout the 28-d course of the study.

To eliminate the possibility that some aspect of virus preparation was eliciting the influx of eosinophils, we repeated this

study, inoculating cotton rats ( $n = 5$ ) with  $10^6$  pfu of virus, either live or UV-irradiated. At day 5 after infection, BAL fluids were analyzed for cell composition. As in saline-treated cotton rats, eosinophils made up 30% of the lavage specimens from cotton rats treated with UV-inactivated virus (Figure 7), with significantly ( $P < 0.05$ ) higher levels in those receiving live RSV. No virus was detected by plaque assay in lung homogenates from cotton rats that received UV-inactivated RSV; the mean titer in cotton rats given live virus was  $8.4 \pm 5.6 \times 10^4$  pfu/g lung tissue.

To ensure that no other aspect of our treatment was causing an eosinophilic response, we performed BAL on 5 untreated cotton rats. These BAL samples contained eosinophils that comprised a mean of  $21.4\% \pm 5.2\%$  of the total cells present; according to a  $t$  test, there was no significant difference ( $P = 0.08$ ) in the

percentage of eosinophils between untreated cotton rats and those inoculated with UV-inactivated RSV. Lungs harvested from the untreated cohort were harvested and examined for any microscopic evidence of pathology. No pathology was evident in any of the 5 cotton rats (Figure 8), but rare eosinophils could be identified in the alveolar spaces.

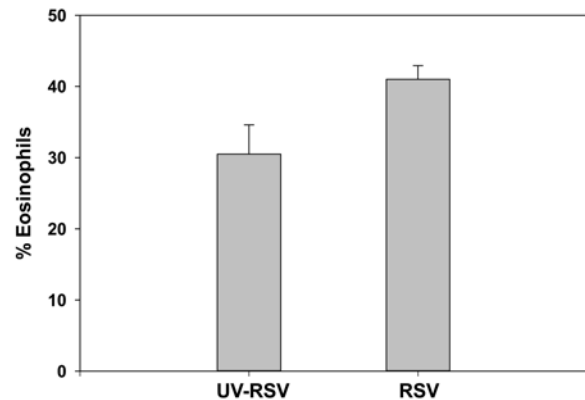
**Asthma-like changes persist in the lungs following clearance of RSV infection.** Severe RSV disease in infancy is increasingly recognized as a major predisposing factor for development of asthma and recurrent wheezing.<sup>53,56</sup> To determine whether RSV infection in cotton rats is associated with the chronic changes found in human patients with allergic asthma, tissues collected 28 d after viral challenge were examined. The histology of the upper airway had returned to normal at this time point, therefore no images from the nasal cavity are shown. In contrast, inflammatory changes persisted in the lower airways (Figure 9).

Chronic changes included mucosal and goblet cell hyperplasia, with persistence of eosinophils in the mucosa and submucosa, and mast cells in the smooth muscle surrounding the airways. In addition, the smooth muscle layer surrounding bronchioles appeared hypertrophied when compared with bronchioles of similar diameter in uninfected rats (Figure 5 A and B), and mucus was present in airways even at the late (day 28) time point. Taken together, these findings are consistent with the chronic changes seen in human patients with asthma and are consistent with the observed association between RSV-induced lower airway disease and later onset of asthma and recurrent wheezing in children.

## Discussion

Our understanding of RSV pathogenesis, and the role played by this ubiquitous virus in the development of allergic responses in the lung, has been limited by the lack of a small animal model that faithfully recapitulates human infection. Mice are the most common animal used to study RSV, but studies using other rodent models and, much less frequently, NHP<sup>11</sup> have been reported. Studies of other pneumoviruses in their primary hosts, including bovine RSV infection of calves and pneumonia virus of mice infection of mice, have been used to gain a better understanding of RSV biology.<sup>38,50,51</sup> The relative merits of these models have been reviewed recently.<sup>4</sup> Each of these systems has unique advantages and disadvantages, but because of the permissivity of cotton rats in regard to RSV replication, this species is increasingly used to evaluate potential RSV vaccines and therapeutics.

Cotton rats are susceptible to a wide range of human pathogens. Although there are, as yet, no genetically modified strains, inbred and microbiologically defined cotton rats are commercially available, as are a growing number of cotton-rat-specific reagents.<sup>7,43,44</sup> Experimental RSV infection of cotton rats was first reported in 1971,<sup>12</sup> and the pathology of RSV-infected cotton rats was first described in 1978.<sup>46</sup> Most subsequent studies of RSV infection in cotton rats have focused on the effect of a specified preventative or treatment on virus replication and clearance, with little attention paid to the pathology.<sup>8,14,33,34,45,47</sup> The source of animals used in one study<sup>12</sup> is not specified, but the colony established in the United States by other authors<sup>46</sup> has been used subsequently by many researchers and is the source of the ani-

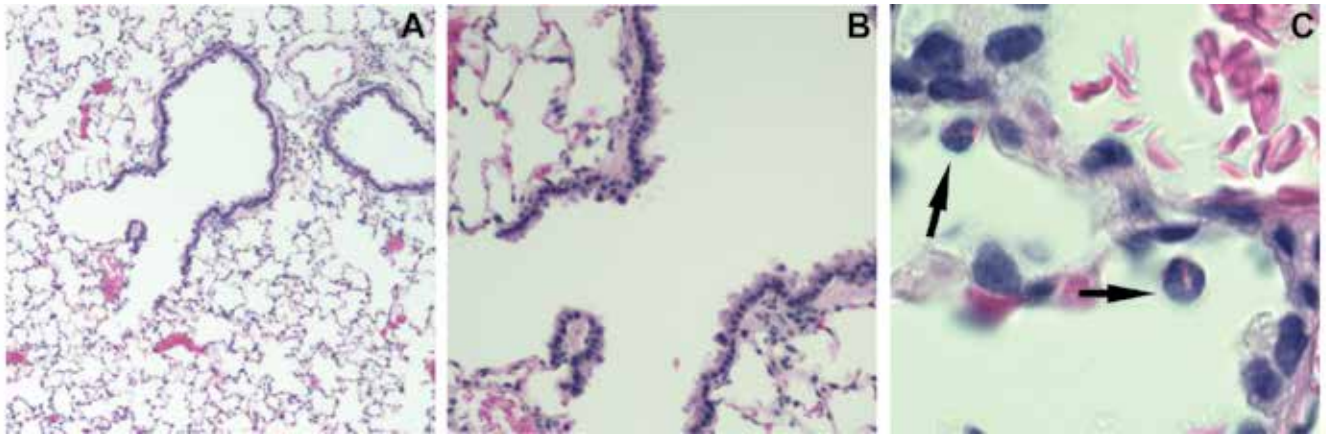


**Figure 7.** BAL cytology in cotton rats treated with UV-inactivated RSV. BAL fluid was collected from cotton rats that received either  $10^6$  pfu RSV, or an equivalent volume of UV-inactivated virus from the same preparation. Samples were harvested at 5 d after inoculation. Differential counts revealed an average of 30% eosinophils in cotton rats given the inactivated virus preparation, consistent with prior values after saline instillation, and an increase ( $P = 0.042$ ) in lung eosinophils in cotton rats that received live RSV. Bars represent the mean value (error bars, SEM).

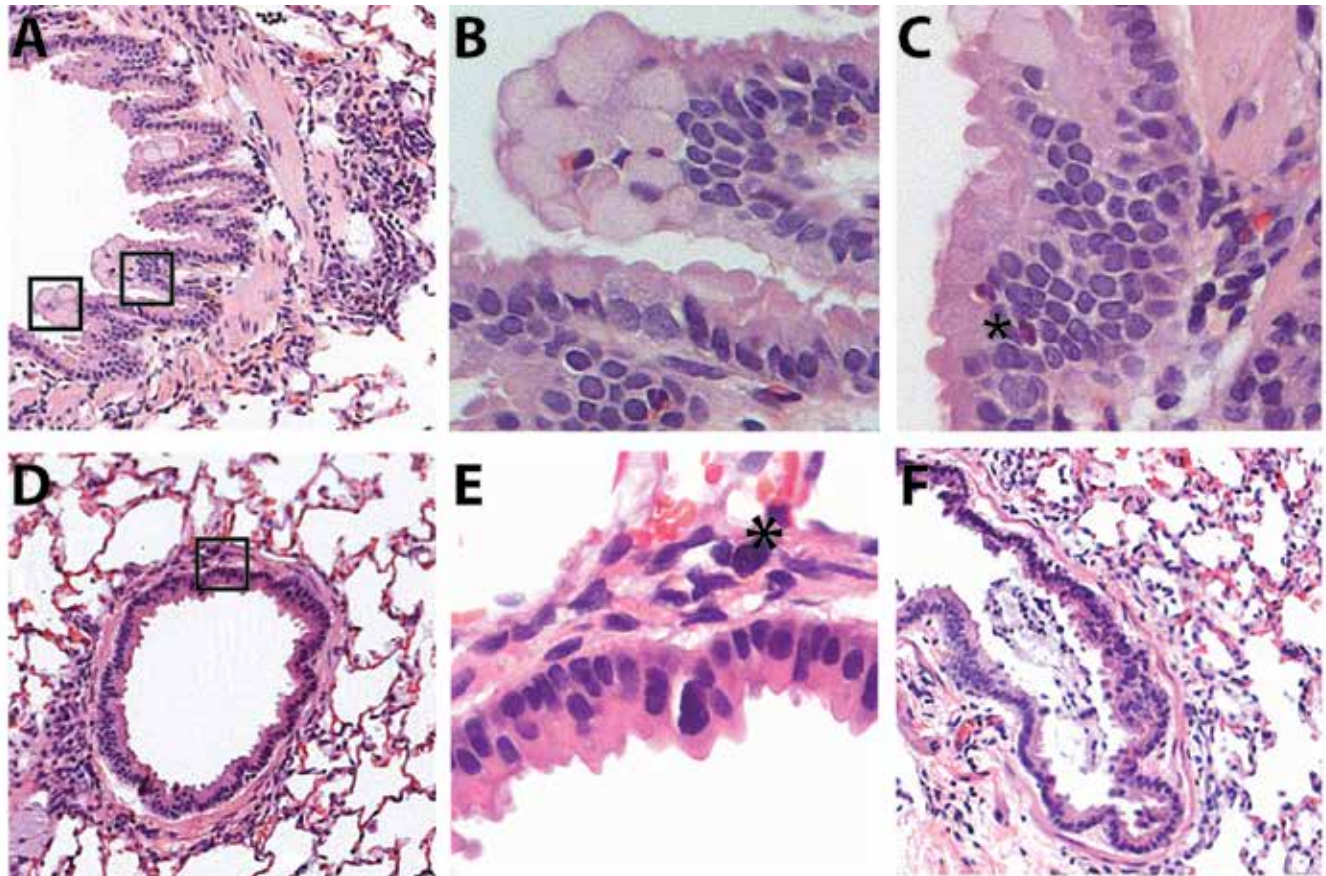
mals we have studied here. This landmark paper<sup>46</sup> showed that, after intranasal inoculation, RSV replicated primarily in the nasal epithelium and in the respiratory epithelium lining the bronchi and bronchioles. Using the strain of cotton rat developed by that group,<sup>46</sup> we have replicated those findings here. This pattern of spread is similar to that described in human patients<sup>32,61</sup> and unlike that in mice, where the viral antigen is largely absent from the nasal cavity and airways but is present in pneumocytes lining the alveolar spaces rather than the airway-lining cells<sup>18</sup> (Figure 10). The inflammatory response to infection described in the earlier study<sup>46</sup> was limited, consisting of intraepithelial neutrophils and a sparse peribronchial and perivascular inflammatory infiltrate made up of lymphocytes and neutrophils. Although the black-and-white photography in the earlier publication<sup>46</sup> make direct comparison difficult, the pathology we have observed is markedly different. These differences may be due to a number of factors, including the use of the A2 rather than the Long strain of RSV, an increased virus dose, the relatively small volume used for inoculation, and the difference in morphology between human and rodent eosinophils. Although eosinophils from both species have brightly eosinophilic cytoplasmic granules, human eosinophils contain a distinctive bilobed nucleus, a feature key to their histologic identification. In mice and cotton rats, eosinophil nuclei are less uniform, often with a donut-shaped nucleus<sup>58</sup> (Figure 6), but remain distinct from the multilobed nuclei of neutrophils.

Here we have described in detail the acute and chronic lung pathology after intranasal RSV A2 infection of cotton rats. After the delivery of low-volume viral inoculum to the nasal cavity, we observed diffuse infection of the uppermost airway by RSV, with more focal involvement of the lungs. This conclusion relies primarily on our immunohistochemistry studies. Virus was detected by plaque assay in both nasal wash and lung homogenate specimens, but direct comparison of those samples tells us relatively little about the spread of infection and the nature of the disease.





**Figure 8.** Eosinophils in the airway of untreated cotton rats with no microscopic evidence of disease. Lungs from 5 untreated rats showed no evidence of infection or inflammation. A representative animal is shown here, with images taken at a magnification of (A) 40 $\times$ , (B) 100 $\times$ , and (C) 600 $\times$ . Rare eosinophils (C,  $\rightarrow$ ) were present in alveolar spaces. The eosinophil is in an alveolar space adjacent to a small vessel filled with RBCs.

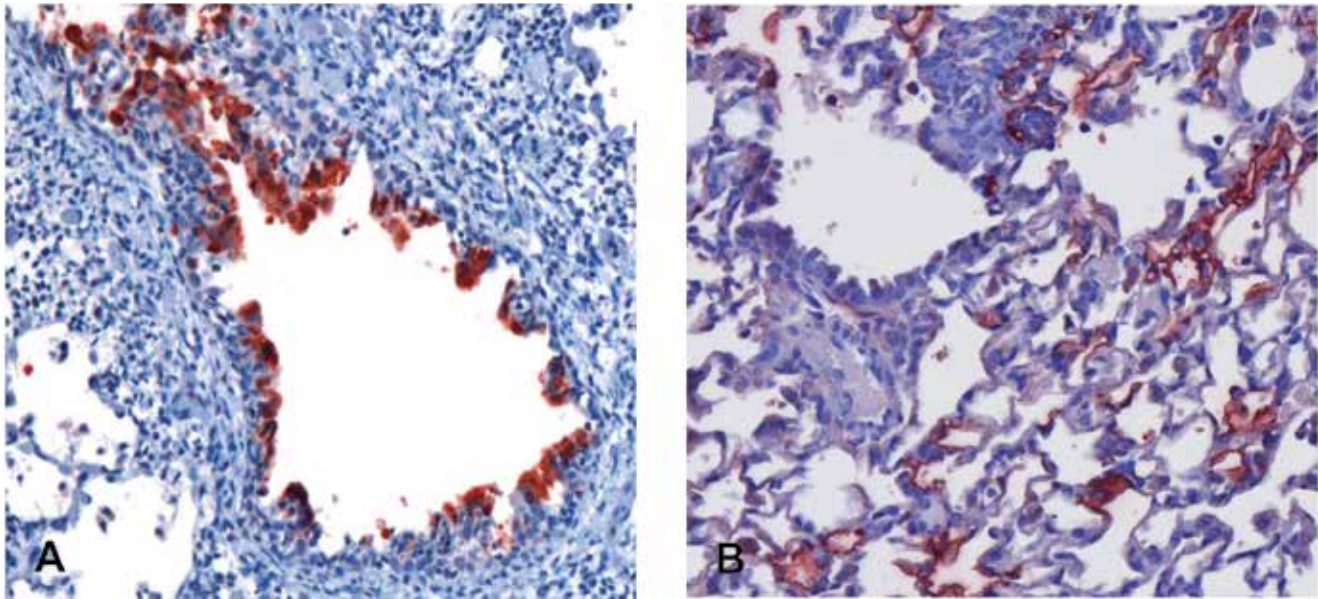


**Figure 9.** Chronic changes in the lung after RSV infection. Lungs were collected 28 d after viral challenge and examined microscopically. (A–F) Chronic changes included persistent peribronchiolar inflammatory infiltrates, (B, C) mucosal and goblet cell hyperplasia, (C) the continuing presence of eosinophils (\*) in the bronchiolar mucosa and submucosa, and (E) the presence of mast cells (\*) in the smooth muscle layer. (F) Mucus was present in terminal airways. Images were taken at a magnification of 100 $\times$  (A, D, E) or 400 $\times$  (B, C, F). Panels B and C show high-power images of fields outlined in panel A. Panel E is a high-power image of the field outlined in panel D.

In our hands, RSV infection of the nose of cotton rats was accompanied by marked acute inflammation accompanied by degenerative epithelial changes. Inflammation persisted at day 7, but by 28 d after infection, an increase in goblet cell number was

the only evidence of prior injury (Figure 4) despite immunohistochemical evidence of prolonged upper airway infection (Figure 3). Acute inflammatory lesions in the lung, limited to the bronchioles and adjacent parenchyma, were more focal and less severe





**Figure 10.** Differing patterns of RSV infection in the lungs of mice and humans. (A) Photomicrograph (magnification, 100 $\times$ ) of a lung lesion in an infant found to have RSV infection at autopsy.<sup>31</sup> (B) RSV replication in the lung of a BALB/c mouse 4 d after intranasal inoculation with 10<sup>7</sup> pfu RSV A2 (magnification, 200 $\times$ ).

and accompanied by epithelial damage and regenerative changes. However, in contrast to that in the nose, lung eosinophilia persisted. Interestingly, even BAL fluid collected from naïve cotton rats that were untreated or challenged only with saline contained a high baseline level of eosinophils, 25% on average. The number of lung eosinophils increased in response to RSV infection and remained elevated until the 28-d point. Chronic changes in lung tissue that remained at 1 mo after infection also included the persistence of eosinophils within the epithelial layer, goblet cell metaplasia, and a thickened bronchiolar smooth muscle layer with infiltrating mast cells. This chronic, RSV-induced, lung pathology recapitulates the chronic changes seen in human patients with asthma.<sup>1,15</sup>

The predominance of eosinophils in BAL fluid and the persistent eosinophilic infiltrates in response to primary RSV challenge of cotton rats were unexpected findings and are contrary to what has been reported in human patients, in whom BAL fluids contain predominately lymphocytes and neutrophils.<sup>32</sup> Despite the prominent eosinophilia in lungs from naïve cotton rats, the number of circulating eosinophils in the blood was not elevated above that in other laboratory species<sup>2,48</sup> or human patients<sup>36</sup> nor is it increased in response to RSV infection (data not shown). The lung eosinophilia we noted here may be a feature of paramyxoviral infection in this species, given that high levels of eosinophils in BAL fluid also occur in cotton rats infected with measles virus.<sup>10</sup> Pulmonary eosinophilia, however, is not known to be a general reaction to virus infection in cotton rats, given that the inflammatory response to the hantavirus Black Creek Canal virus, a natural pathogen of cotton rats, is mediated by mononuclear cells.<sup>5</sup> Whether other pathogens induce an eosinophilic response in this species is unknown, and in the absence of specific pathogen testing for cotton rats, the possibility of intercurrent infection cannot be eliminated.

Human studies of RSV infection are limited by safety issues and the fact that, despite its ubiquity, RSV infection does not

usually result in severe disease. Therefore, relatively little clinical or autopsy material is available for study and none from patients with the typical, mild course. Published studies focus on differences between more and less severely ill, hospitalized patients, thus yielding relatively little insight into the mechanisms that, although insufficiently effective to prevent infection, can limit spread. RSV infections in the majority of in human patients are limited to the upper airway,<sup>9</sup> with only 40% to 50% of primary infections in infants resulting in clinically significant lower airway disease.<sup>55</sup> Therefore animal models of RSV pathogenesis that, unlike mice, are susceptible to infection of the uppermost airway are urgently needed. We note that RSV infection of cotton rats shows a pattern of virus growth and spread similar that found in human patients, although no clinical signs of illness were apparent in cotton rats. In addition, the virus-mediated development of asthma-like pathology in cotton rats, even in the absence of severe disease, may represent a new opportunity to study the connection between RSV infection and the development of allergic asthma.

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