JOURNAL OF VIROLOGY, May 2005, p. 6035–6042 Vol. 79, No. 10
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.10.6035–6042.2005

Chinchilla and Murine Models of Upper Respiratory Tract Infections with Respiratory Syncytial Virus

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Received 4 August 2004/Accepted 9 January 2005

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infections in infants and the elderly. While the primary infection is the most serious, reinfection of the upper airway throughout life is the rule. Although relatively little is known about either RSV infection of the upper respiratory tract or host mucosal immunity to RSV, recent literature suggests that RSV is the predominant viral pathogen predisposing to bacterial otitis media (OM). Herein, we describe mouse and chinchilla models of RSV infection of the nasopharynx and Eustachian tube. Both rodent hosts were susceptible to RSV infection of the upper airway following intranasal challenge; however, the chinchilla proved to be more permissive than the mouse. The chinchilla model will likely be extremely useful to test the role of RSV in bacterial OM and the efficacy of RSV vaccine candidates designed to provide mucosal and cytotoxic T-lymphocyte immunity. Ultimately, we hope to investigate the relative ability of these candidates to potentially protect against viral predisposal to bacterial OM.

Many factors contribute to the prevalence of middle ear infections in children as well as to the chronic or recurrent nature of otitis media (OM). These include immunological immaturity, existence of other infections, anatomic positioning of the Eustachian tube (ET), and genetic predisposition (10, 14, 22). However, whereas the multifactorial nature of OM is well known, it has only recently become fully appreciated that both acute and chronic OM are truly polymicrobial infections, involving any of several upper respiratory tract (URT) viruses and one or more of three bacterial pathogens.

There is now ample epidemiological evidence and data generated by PCR-based assays, immunoassays, and direct culture to support the association of URT viruses with acute bacterial OM (2, 3, 15, 16, 39, 46–50, 55, 60, 64, 67–69). Moreover, peak incidence of OM occurs in concert with peak periods of viral isolation (38, 56, 64). Importantly, exposure to URT viruses (primarily via day care attendance or association with siblings) is a significant risk factor and/or predictor for early onset, frequent, or recurrent OM (51, 58). In a prospective study of 596 infants, Daly et al. (20) found that exposure to URT viruses is indisputably the single most important predictor for early acute OM. While the crucial role of URT viruses in the pathogenesis of bacterial OM is now firmly established (24, 32–36, 64, 65), we do not yet have a complete understanding of the mechanisms involved.

Nearly all respiratory tract viruses can predispose to bacterial OM, but different viruses, and even different strains of the same virus, can differ in their relative ability to do so. The degree to which a particular virus compromises the airway, particularly the ET, has a tremendous influence on the occurrence and severity of OM (28, 77). Whereas the cited studies describe intrastrain variability in the ability of influenza virus to predispose to OM, there are also ample data to suggest that other viruses are more commonly associated with OM, particularly respiratory syncytial virus (RSV) (35, 37, 39, 46, 47, 49, 53, 61, 64, 66, 73).

Thus, in addition to causing secondary URT infections throughout life, RSV is a key viral copathogen of OM. Despite its importance, the specific mechanisms by which RSV predisposes to OM have not been studied, largely due to the absence of a relevant animal model. While many animals can be infected with RSV, following direct inoculation of the respiratory tract, only humans and chimpanzees are highly permissive for human RSV replication. Several animal models of RSV-induced infection of the lower respiratory tract exist (13, 18, 19, 54), but RSV pathogenesis in the uppermost airway has not been extensively studied. Very little virus has been detected in the nasal cavities of mice infected intranasally (i.n.) (30), although RSV infection of nasal mucosa has been shown in the more permissive cotton rat (63).

Herein we present evidence that murine and chinchilla hosts are susceptible to nasopharyngeal (NP) and ET infection by RSV following intranasal challenge. In addition, we have observed that chinchillas, unlike mice, are relatively permissive for RSV infection, showing signs of illness in addition to evidence of virus replication and virus-induced inflammation. These rodent models will provide useful and relevant in vivo systems to assay both the pathogenesis of and the host immune response to RSV infection, as well as provide models in which

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to assay the relative efficacy of potential RSV vaccine candidates.

MATERIALS AND METHODS

Virus preparation and assay. High-titer stocks of RSV (A2 strain) were prepared on HEp2 cell monolayers by modification of published methods (30). In order to detect low levels of bacterial or mycoplasmal contamination, cultures were maintained in the absence of antibiotics. Virus titers were determined by plaque assay on STAT1$^{-/-}$ mouse fibroblasts (23). Equivalent numbers of plaques were counted on STAT1$^{-/-}$ fibroblast and HEp2 monolayers, but they appeared much more rapidly on the knockout murine cells: 36 h versus 5 days, respectively (data not shown).

Animals. Nine juvenile chinchillas (*Chinchilla lanigera*) (mean weight, 399 ± 74 g) were purchased from Rauscher's Chinchilla Ranch (LaRue, OH) and used for these studies, after resting for 7 to 10 days. Juvenile chinchillas, approximately 3 months of age and weighing 300 to 350 g, were used here due to our previous demonstration of the preferred use of juvenile chinchillas when demonstrating adenovirus predisposition to bacterial invasion of the middle ear (8, 45). Male and female BALB/c mice were obtained from Harlan Laboratories (Indianapolis, IN) and were infected at 6 to 10 weeks of age.

Infection. Mice were infected i.n. following methodologies that were developed to deliver an inoculum specifically to the upper airway using a very small volume (43, 76). Briefly, each mouse was lightly anesthetized, placed in a supine position, and given 10^7 PFU of RSV in a 20-μl volume. Two microliters per naris were delivered at 0, 2, 7, 9, and 11 min. There were three mice per data point.

Chinchillas were also inoculated i.n. with RSV, again delivered specifically to the upper airway (76). The dosages (in total PFU delivered), 10^3, 10^5, 10^6, or 10^7, were administered by passive inhalation of droplets of viral suspension delivered to the nares of chinchillas that were lightly anesthetized with xylazine (2 mg/kg of body weight; Fort Dodge Animal Health, Fort Dodge, IA) and ketamine (10 mg/kg; Phoenix Scientific Inc., St. Joseph MO) and were laying in a prone position. Initially, we used three chinchillas to attempt to identify the dose of RSV that would induce maximal compromise of Eustachian tube function. Thus, one animal received a dose of 10^3, 10^5, or 10^7 PFU of RSV for this early assessment. Subsequently, to better define RSV-induced histopathology and determine the relative reproducibility of RSV-induced upper airway compromise in this host, six additional juvenile chinchillas were inoculated with either 10^6 or 10^7 PFU RSV (3 chinchillas/dose). No chinchillas were inoculated with either sterile saline or sterile tissue culture medium in the present study due to the existing extensive evaluation of the normal histology, Eustachian tube function, and microbial flora resident in this host under these control conditions in the literature (6, 7, 9, 17, 26, 27, 44, 52, 59, 72).

Otoscopy and tympanometry. Chinchillas were evaluated daily, with signs of tympanic membrane inflammation determined by video otoscopy (Video Vet-Scope system; MedRx, Seminole, FL) and rated on a 0 to 4+ scale, as described previously (8, 45, 76). Tympanometry (EarScan, South Daytona, FL) was used to monitor changes in middle ear pressure, tympanic width, and tympanic membrane compliance, also as described previously (8, 45).

Lavages and recovery of tissues for quantitative viral culture. RSV-infected mice and uninfected controls were sacrificed at various times postinfection by CO_2 inhalation. For virus titration, lungs and nasal mucosa were immediately frozen in liquid nitrogen. Prior to plaque assay, tissues were weighed and homogenized in 1 ml phosphate-buffered saline (PBS). Tissue homogenates were assayed as described above.

In chinchillas, middle ear lavages were performed on day 4 post-RSV inoculation by first venting the superior cephalid bulla with a 25-gauge needle and depositing 300 μl of pyrogen-free sterile saline into the middle ear cavity. Lavage fluids were then recovered from the middle ear by "epitympanic tap," which involved withdrawing these fluids from the middle ear cavity via use of a 1.5-in., 25-gauge hypodermic needle. NP lavage was also performed on days 4 or 8 postinoculation of RSV by passive inhalation of 500 μl pyrogen-free sterile saline. Lavage fluids were collected as they drained from the contralateral naris and snap-frozen in liquid nitrogen until used for determination of viral titer. As per our usual protocol, in all cases epitympanic taps were performed prior to NP lavage to limit the possibility of introducing commensal bacteria into the tympanum via refluxing of lavage fluids.

Lung and tracheal tissues were recovered on day 4 after RSV inoculation, whereas NP and ET mucosae were recovered on days 4 or 8. Middle ear lavage fluid, lungs, and tracheal mucosa retrieved on day 4 were from a single animal that received 10^7 PFU, whereas all other specimens were retrieved from three chinchillas per dose of either 10^6 or 10^7 PFU RSV. All fluids and tissues were snap-frozen in liquid nitrogen.

Serum neutralization assay. RSV (500 PFU/ml) was mixed with serial dilutions of chinchilla serum and incubated at room temperature for 30 min. These mixtures (100 μl) were used to inoculate confluent monolayers of mouse STAT1$^{-/-}$ fibroblast cells grown in 24-well plates. After a 1-h incubation at 37°C, infected wells were washed and refed with growth medium plus 0.5% methylcellulose. After 2 days, plates were stained with crystal violet and plaques counted. The neutralizing titer was defined as the serum dilution that resulted in a 50% reduction in plaque number compared to controls that had no serum.

Histology. Sections through the NP and ET of mice were obtained following formalin fixation and decalcification. For immunohistochemistry, sections were blocked with a Vector Avidin/Biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA) and Super Sniper (Biocare Medical, Walnut Creek, CA) before overnight incubation at 4°C with goat anti-RSV antibodies, 1:500 (Biodesign, Saco, ME). Staining was visualized using a biotinylated mouse anti-goat secondary (ScyTek, Logan, UT) antibody, streptavidin-horseradish peroxidase (Biocare Medical), the chromogen AEC (DAKO, Carpinteria, CA), and a hematoxylin counterstain.

For indirect fluorescence microscopy, ETs recovered from chinchillas inoculated with 10^7 PFU of RSV were embedded in low-temperature embedding medium or cryocompound (Fisher Scientific, Pittsburgh, PA) and cryosectioned. Sections (5 μm) were fixed with cold 3.5% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), washed, blocked with 0.5% ovalbumin in PBS, and incubated 2 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-RSV (Biodesign International, Saco, Maine). FITC-conjugated goat immunoglobulin (Ig) (Caltag Laboratories, Burlingame, CA) was used as a non-RSV specific control antiserum and Evans blue was used on all tissues as a counterstain (this dye will fluoresce red under the conditions used to image FITC labeling, which appears as green fluorescence). Images were captured using a Zeiss LSM 410 confocal microscope (Carl Zeiss, Thornwood, NY).

RESULTS

Murine susceptibility to RSV infection of the NP and ET following i.n. challenge. BALB/c mice have been widely used to study lower respiratory tract infection by RSV following i.n. inoculation, but titers from mouse nasal cavities are generally very low, on the order of 100 to 1,000 PFU/nose. Similar results have been reported by others (30). Here, we examined the course of NP and ET infection by RSV in BALB/c mice by immunohistochemical methods, looking for cells expressing RSV antigens rather than scoring virus titers by plaque assay. An important departure from previous studies (21) was our method for inoculation (43, 76) using a concentrated virus suspension and delivering the inoculum in multiple small aliquots over a 20-min interval. Unlike typical i.n. protocols that use larger (50 to 100 μl) volumes, this method allows the inoculum to be absorbed by the nasal mucosa rather than being delivered through the nose into the trachea and esophagus.

Histologic examination was performed on sections from the NPs and ETs of BALB/c mice at days 4, 7, and 10 after RSV infection. Transverse sections, or cross sections, through the nasal cavity were taken at the levels used routinely in toxicology studies. The mucosal lining of the nasal turbinates and septum is primarily columnar respiratory epithelium at the level of the incisors (level I), transitioning to primarily olfactory epithelium at the level of the second molar (level III) (74). A transverse section taken at level III from an uninfected mouse is pictured in Fig. 1A. Superiorly is the bony skull to which the centrally located nasal septum is attached. At the lower aspect is the palate; just above the palate is the septal window with two triangular shaped masses of nasal-associated lymphoid tissue positioned at its ventrolateral edges. Ciliated respiratory epithelium lines the ventral portion of the nasal cavity and the maxillary sinus. Sections from infected animals

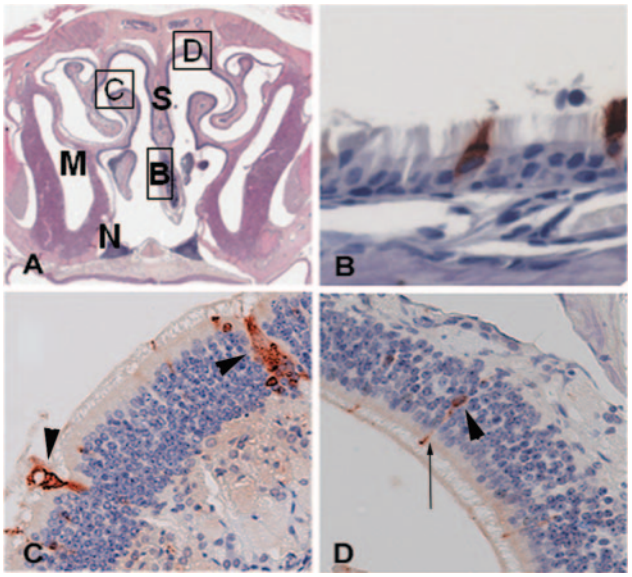

FIG. 1. Panel A is a low-power (15×) H&E-stained section showing a transverse section, or cross section, of the murine nasal cavity taken at the level of the second molar. At the superior aspect of the specimen is the bony skull to which the nasal septum (S) is attached. The palate is present at the lower aspect, and just above the palate are the two triangular masses of nasal-associated lymphoid tissue (N). M, maxillary sinus. Immunostained respiratory mucosa lining the nasal septum (B), olfactory mucosa lining the turbinate (C), and the dorsal surface of the nasal cavity (D) are shown in panels B, C, and D, respectively. All specimens shown are from day 4 postinoculation. In panel B, red-brown immunostaining is seen at the apical surface of one ciliated cell and throughout the cytoplasm of a second cell (400×). In panel C, two ducts from Bowman's glands are staining strongly with the anti-viral antiserum and are marked with arrowheads (200×). In panel D, cell bodies of olfactory neurons (arrowhead) and their dendritic processes (arrow) are infected with RSV (200×).

were evaluated by staining for RSV antigen expression by using polyclonal whole-virus antisera. Infected cells could be visualized at day 4 when virus production is near or at peak levels (21) (Fig. 1). Figure 1B shows RSV infection of ciliated respiratory epithelial cells lining the nasal septum. The red-brown immunostaining appears either apically or extends throughout the cytoplasm. This staining pattern is consistent with the biology of RSV that is known to infect through, and bud from, the luminal surface of respiratory epithelial cells (18). The images shown are representative of multiple sections from each of three animals from each genotype at this time point.

The tropism of RSV for respiratory epithelium is well established; however, a surprising and intriguing result from these studies was its equal affinity for the olfactory mucosa (Fig. 1C and D). Olfactory mucosa lines the ethmoid turbinates, the dorsal wall of the nasal cavity, and the dorsal portion of the nasal septum at level III. The olfactory epithelium consists of the supporting sustentacular cells, bipolar olfactory neurons, and the excretory ducts from Bowman's glands. Sustentacular cells are tall columnar cells, extending from the basal lamina to the lumen. They have microvilli on their apical surface and their nuclei form a layer occupying the upper one-third of the olfactory mucosal layer (40). Morphological analysis clearly shows RSV infection of the ductal epithelium in Fig. 1C. Less prominent, but clearly seen, are infected bipolar neurons with antibody staining of their axons and dendrites (Fig. 1C and D). These findings were consistent over the course of three experiments, and staining of olfactory mucosa was noted in the chinchilla as well.

Murine ETs were removed at 4, 7, and 10 days, formalin fixed, and extensively decalcified. Longitudinal sections of both infected and uninfected ETs were prepared and examined (Fig. 2A). Both ends of the murine ET are lined by pseudostratified, ciliated mucosa intermixed with goblet cells (Fig. 2B, C, and D). In all mice examined, infected or uninfected, a sparse inflammatory infiltrate consisting of lymphocytes and eosinophils was present in the lamina propria underlying the nasopharyngeal ET opening. The mid-portion of the ET tube is lined by cuboidal, sparsely ciliated epithelium (Fig. 2B). Figure 2D shows RSV infection of a single ciliated cell present in the distal ET at the opening to the middle ear, with the red-brown immunostaining concentrated at its apical, ciliated surface. Only rare, single infected cells were identified among six animals examined at day 4 postinfection, and no virus-infected cells were identified after day 4. Pathological changes in both the NP and ET were subtle. The most notable change was the accumulation of acute inflammatory cells (mostly neutrophils and macrophages) and mucus in the ET lumen (Fig. 2C) at day 10. Other inflammatory changes in the NP and ET were mild, with a small increase in inflammatory cells within the lamina propria and mild goblet cell hyperplasia. These findings are representative of three animals at each time point in each of two experiments.

Chinchilla permissivity to infection of the upper airway with RSV following i.n. challenge. A dose-effect was clearly detectable in chinchillas inoculated with increasing doses of RSV. At the lower dosages assayed (1×10^3 and 1×10^5 PFU/animal), no signs of illness were noted at any time postchallenge. Conversely, animals that received higher dosages (e.g., 1×10^6 and 1×10^7 PFU/animal) showed signs of acute respiratory tract infection. Ruffling of fur and lethargic behavior were observed by day 4 after challenge in 2 of 6 (33%) animals that received the 1×10^7 PFU inoculum; however, the hallmark sign of infection was mild retraction of the tympanic membrane as measured by both otoscopy and tympanometry. By otoscopy, there was evidence of low-grade inflammation of the tympanic membrane (maximum value, ~1.5 on a 0 to 4+ scale), typical of viral OM, with retraction being the most consistent sign of RSV infection in this host at a dose of 10^7 PFU.

Retraction was noted as early as 1 day after i.n. challenge in chinchillas inoculated with 1×10^7 PFU RSV. In animals that received either 1×10^6 or 1×10^7 PFU, retraction was maximal 3 to 4 days after challenge, occurring in 67 to 100% of ears as noted by otoscopy and persisting until at least 9 days after inoculation, although the degree of retraction was less marked at day 9 (data not shown) than that noted on days 3 to 4. In these animals, evidence of retraction was supported by demonstration of an underpressured state (Fig. 3A) occurring in 63 to 88% of ears that was also maximal 3 to 4 days after challenge (Fig. 3B). Middle ear pressure steadily declined after inoculation of RSV, with a mean maximal underpressured middle ear state of −45 daPa occurring approximately 4 days after i.n. challenge with 10^7 PFU RSV. This underpressured state persisted until at least 7 days after inoculation, although both degree of underpressure and number of ears affected was

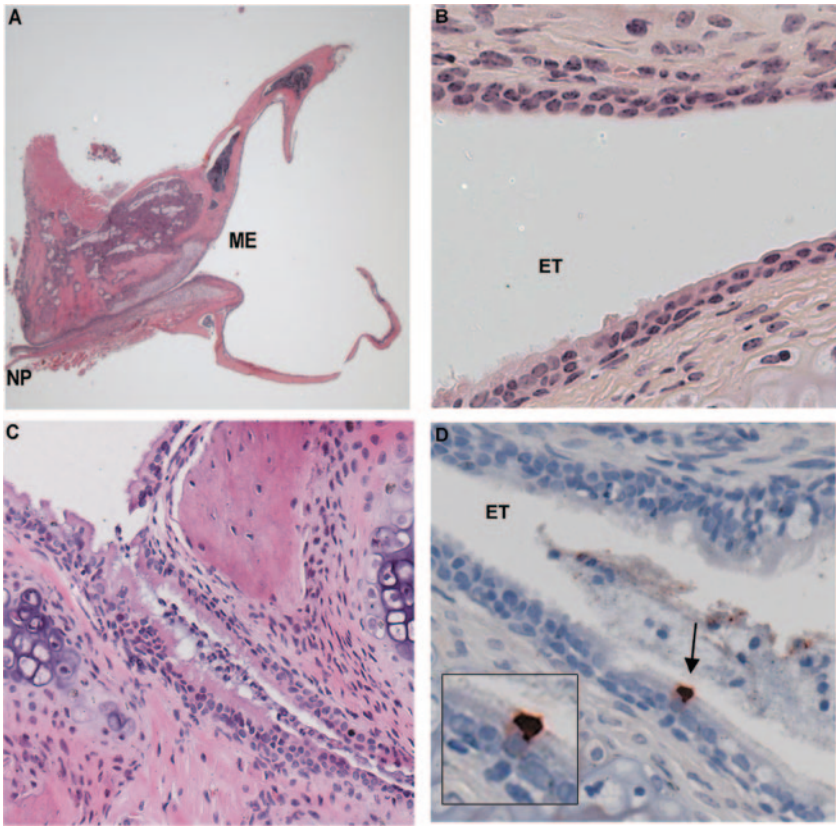

FIG. 2. Panel A is a low-power (15×) H&E-stained section showing an entire ET dissected from an uninfected mouse. On the left side of the image is the NP mucosa at the opening of the ET. The ET opens distally into the middle ear (ME) cavity, which can be seen at the right side of the section. In panel B (400×), ciliated mucosa at the proximal, nasopharyngeal end of the ET gives way to a more flattened epithelial cell type. A section from the ET of a RSV-infected mouse (panel C) shows the accumulation of mucous and inflammatory cells in the lumen at day 10 after inoculation (200×). Staining with anti-RSV antiserum shows that ciliated ET epithelium can be infected. The cell shown in panel D is at the distal end of the ET (400×), where it opens into the middle ear cavity.

markedly reduced at this time point. Together, these data suggested RSV-induced ET compromise. In addition, nasal lavage fluids recovered from several RSV-infected chinchillas had an abnormal yellowish-green tint and were notably turbid. This latter observation is consistent with histopathological evaluations that showed hypersecretion of mucus into the ET lumen in tissues recovered from chinchillas inoculated with RSV (see below).

Plaque assays conducted with homogenized NP mucosa and NP lavage fluids indicated that the chinchilla was permissive to RSV infection (Table 1). Both NP mucosal tissue homogenates and NP lavage fluid specimens were positive on days 4 and 8 after challenge at viral doses of $\geq 10^5$ PFU. Preliminary evidence in support of restriction of viral replication to the uppermost airway following i.n. challenge in the manner described here was supported by the absence of viral plaques when tracheal mucosa and lung tissue recovered 4 days after challenge from a single chinchilla that had received the highest dose of RSV (10^7 PFU) were homogenized and assayed for RSV via plaque assay. More extensive evaluation of this phenomenon is needed.

RSV infection results in goblet cell hyperplasia, hypersecretion of mucus, and the clear presence of RSV antigen within cells lining the ET. Examination of hematoxylin and eosin (H&E)-stained sections of NP and ET mucosae recovered from chinchillas infected i.n. with RSV revealed signs of mild inflammation. There was a sparse mononuclear submucosal infiltrate with eosinophils proximally. Marked goblet cell hyperplasia with mucus hypersecretion into the lumen of the ET was however a consistent finding in RSV-infected chinchillas, as evidenced by periodic acid-Schiff–Alcian Blue staining which is specific for mucopolysaccharides present in these tissues (Fig. 4). Note relative the abundance of mucus in the Eustachian tube lumen, the number of heavily stained goblet cells, and the intensity/character of staining of submucosal glands in an RSV-infected chinchilla versus a noninfected control animal when comparing panels A and B versus C and D, respectively, in Fig. 4. By using a biotin-conjugated goat anti-RSV polyclonal antiserum, we showed the presence of RSV antigen in ciliated cells lining the chinchilla ET at various or multiple points along approximately the proximal two-thirds of its length (similar to that seen in Fig. 2D), thus demonstrating the ability of RSV to infect the chinchilla ET following i.n. inoculation. When frozen sections of whole chinchilla ETs (including tissues from the pharyngeal to tympanic orifice), recovered from animals challenged with RSV, were incubated with FITC-conjugated goat IgG antibody, there was no fluorescent labeling (Fig. 5A). Conversely, tissues incubated with

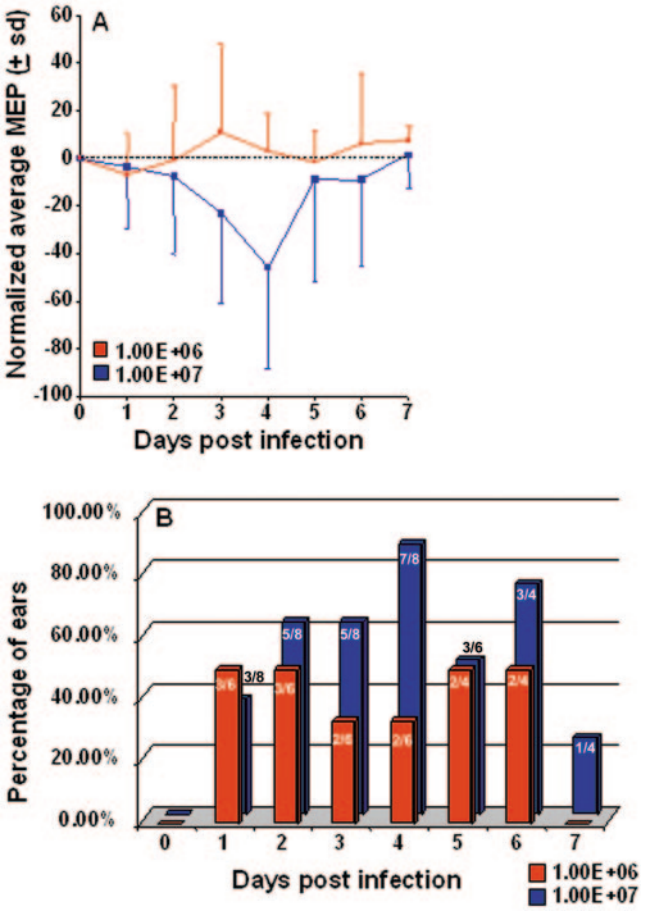

FIG. 3. Panel A, changes in normalized average middle ear pressure (± standard deviations [sd]) over time in chinchillas infected with either 10^6 or 10^7 PFU RSV strain A2 via intranasal delivery. Middle ear pressure (MEP) was assessed by tympanometry. Panel B, relative percentage of ears that demonstrated reduced middle ear pressure relative to baseline measurements over time in cohorts that received either 10^6 or 10^7 PFU RSV. Values on bars indicate the number of ears demonstrating a reduced middle ear pressure over the total number of ears evaluated.

FITC-conjugated goat anti-RSV showed distinct fluorescent labeling of the epithelial lining for three quarters of the length of the ET from the NP to the tympanic orifice (Fig. 5B). Control tissues from naive animals did not label with FITC-conjugated goat anti-RSV antibody (data not shown).

TABLE 1. RSV titers from chinchilla NP mucosa and lavage fluids

Viral dose	RSV titer[a]			
	Log PFU/g NP mucosa		Log PFU/ml NP lavage fluid	
	Day 4	Day 8	Day 4	Day 8
10^5	3.4	ND[b]	3.15	ND
10^6	3.9	2.4	2.9	2.3
10^7	3.6	3.6	3.6	2.6

[a] The data presented are mean titer/group. One chinchilla received 10^5 PFU, three received 10^6 PFU, and four animals received 10^7 PFU.
[b] Not done.

Further evidence that the chinchilla host was permissive to RSV infection was provided by the demonstration of development of virus-neutralizing antibody in sera recovered from these animals. No plaque-neutralizing antibody was observed in sera recovered from either five chinchillas prior to challenge or in two chinchillas 8 days after i.n. delivery of either 10^6 or 10^7 PFU RSV. However, 30 days after receiving either of these latter doses of RSV, plaque-neutralizing antibody with a 50% plaque reduction assay of 1/160 was present in the sera of both animals assayed. To the best of our knowledge, this is the first published report of a chinchilla model for RSV infection of the uppermost airway.

DISCUSSION

As most studies have focused on RSV infection of the lower respiratory tract, relatively little is known about RSV disease of the upper airway, including the NP, ET, and middle ear. To better understand URT infection with RSV and predisposition to bacterial OM by RSV, we need useful and relevant animal models in which to assay pathogenesis and the host immune response to viral infection. Ultimately, animal studies will be necessary to assess the relative efficacy of potential vaccine candidates directed against RSV. Whereas there are several established models of lower airway infection (13, 18, 19), RSV infection of the uppermost airway has not been extensively studied. Barely detectable virus titers were noted in the murine nasal cavity accompanying pulmonary infection (30). Cotton rats infected i.n. with RSV were more permissive for nasal infection, with titers on the order of 10^4 PFU/g (63). Despite their limitations, both mouse and cotton rat models are very useful for the study of possible immunopathology accompanying delivery of any vaccine candidate; however, mechanistic studies have been most informative in the mouse due to the abundance of available murine reagents (1, 29, 41, 57, 71). Nevertheless, at present, little is known about viral OM in the mouse or cotton rat.

In the studies described here, using BALB/c mice, we have determined that the respiratory and olfactory mucosae of the nasal cavity and the ciliated epithelium of the ET can be infected by RSV. Nonetheless, only a small number of infected cells can be detected, and there is very little pathology accompanying infection. In response to infection, submucosal lymphocytes and eosinophils are mildly increased in the NP and at the entrance to the ET. Mucous and inflammatory cells were found along the length of the ET between 7 and 10 days.

In contrast to these observations, we have established that the chinchilla, the preferred rodent host for OM research (25, 31), is susceptible to RSV infection. Chinchillas infected i.n. with RSV showed milder signs of illness than those shown when inoculated similarly with either influenza A virus or with adenovirus (26, 28, 72). Nonetheless, these animals were manifesting signs of URT infection, and these observed signs of illness were dose dependent. RSV-induced compromise of chinchilla ET function was clearly demonstrable. While the underpressured state induced was not marked, it was nonetheless present and concordant retraction of the tympanic membrane was observed. Moreover, the noted goblet cell hyperplasia within the ET mucosa and increased mucus secretion into the ET lumen could provide additional means to predispose to

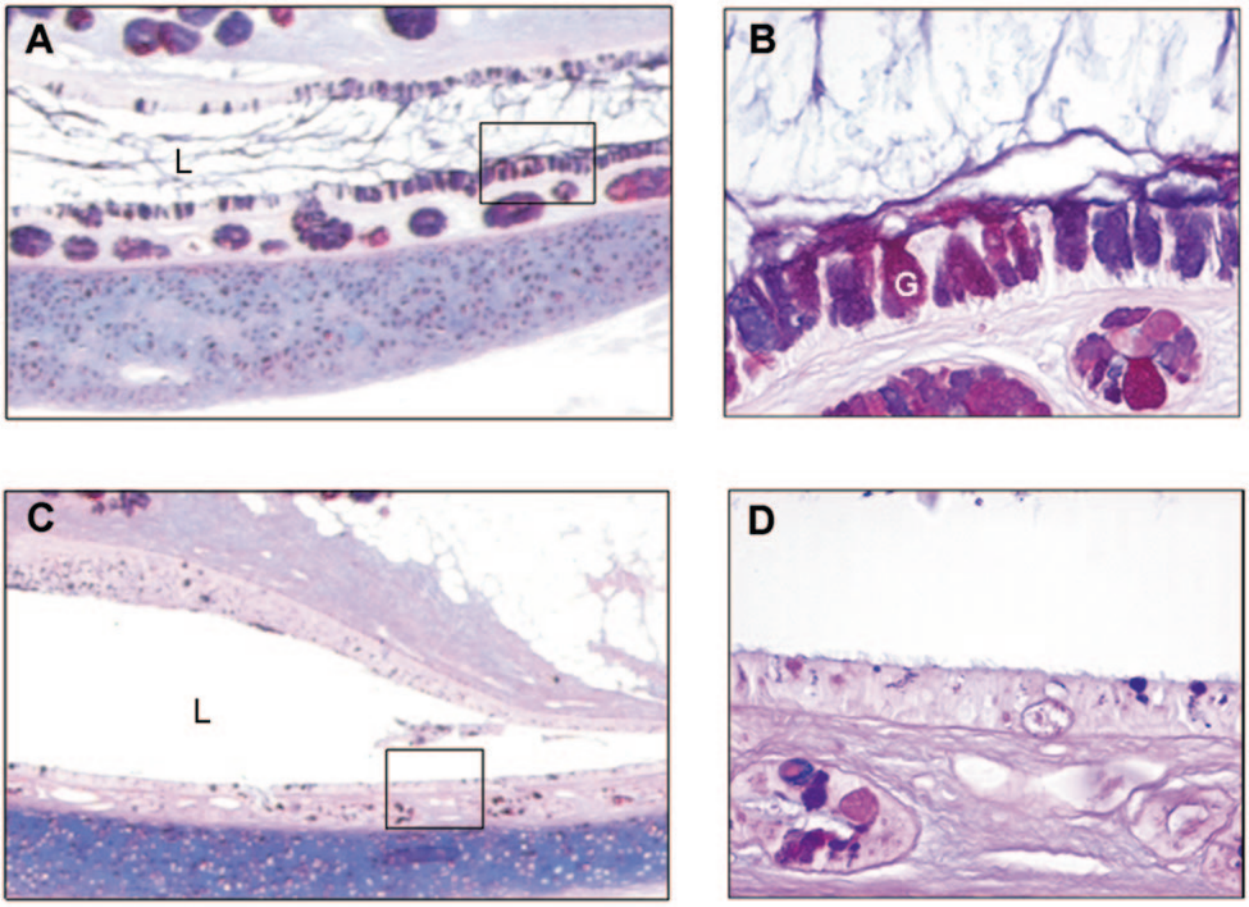

FIG. 4. Periodic acid-Schiff–Alcian Blue staining of mucopolysaccharides present within Eustachian tube tissues showing goblet cell hyperplasia with mucus hypersecretion into the lumen was a consistent finding in RSV-infected chinchillas. Panel A, (~5×) mid-Eustachian tube of a chinchilla 4 days after inoculation with 1×10^7 PFU RSV. Note goblet cell (see representative goblet cell labeled with a white letter "G") hyperplasia relative to noninfected chinchilla as shown in panel C. Inset in panel A is shown at ~40× in panel B; note abundant mucus in ET lumen (labeled "L") and prominent labeling of both goblet cells and glandular structures, submucosally. Inset in panel C is shown at ~40× in panel D; note relative paucity of goblet cells and notably diminished staining of lumenal mucus (Eustachian tube lumen is labeled "L") and submucosal glands in this ET, recovered from a non-RSV-infected animal, when compared to inset panel B.

bacterial superinfection of the tympanum. These phenomena, as well as others, have been shown to be associated with both adenovirus and influenza A virus predisposition to bacterial invasion of the middle ear in the chinchilla (26, 28, 72) and thus parallel the disease course in children, wherein bacterial OM is a highly prevalent complicating sequela of viral URT infection (36).

While both of these rodent models will likely be extremely useful for defining RSV pathogenesis and the host's immune response to infection of the upper airway, each has distinct advantages. The availability of a large range of immunological reagents in the murine system will allow us to characterize virus-specific T- and B-cell responses to primary and secondary nasopharyngeal RSV infection in naive and vaccinated wild-type animals. Our demonstration that RSV infection of the upper airway can be monitored histologically will allow for the use of BALB/c mice in challenge experiments, despite the low virus titers found in the murine nasal cavity.

On the other hand, the chinchilla is a well-established model for OM research due to its larger size, its susceptibility to the microorganisms that cause OM, and the significant parallels that exist between the course of combined viral-bacterial disease induced in chinchillas and the course of natural human disease (4, 5). The chinchilla model will thus provide the first experimental system where the predisposition to bacterial OM by RSV might perhaps be rigorously studied. Despite the fact that, when compared to humans and chimpanzees (18), even the chinchilla is relatively nonpermissive for RSV, rodent models of RSV infection may still offer the most practical solution regarding the need for small and inbred animals to use in proof-of-concept studies and preclinical vaccine candidate trials relevant to the prevention of RSV-induced disease.

In summary, the field of RSV research to date has largely been focused on strategies to prevent life-threatening RSV-induced bronchiolitis and pneumonia in the infant, with little attention paid to protection against upper airway infection. However, it is the upper airway that is continually reinfected, and studies have shown that serum IgGs, though able to prevent lower airway infection, do not constitute effective protection against upper airway disease (42, 62, 70). Immunization

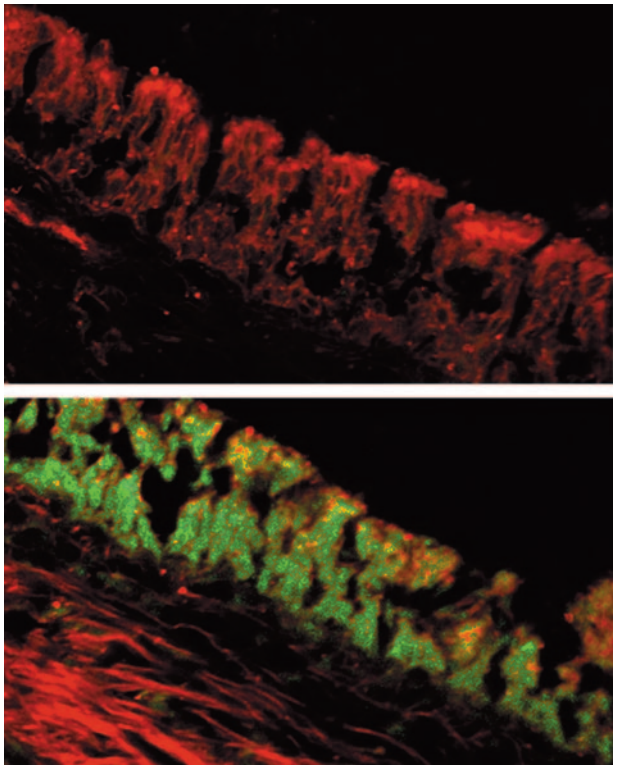

FIG. 5. Fluorescent microscopy images of frozen sections of whole chinchilla ETs recovered from a chinchilla 4 days after intranasal challenge with 10^7 PFU RSV. There was no fluorescent labeling when these tissues were incubated with the control antiserum, FITC-conjugated goat IgG (top panel) or when non-RSV-infected control tissues were labeled with FITC-conjugated goat anti-RSV antibody (not shown). Conversely, distinct green fluorescent labeling of the mucosal epithelium lining this tubal organ was seen when frozen ET sections were incubated with FITC-conjugated goat anti-RSV antibody (bottom panel). Red color in tissues is due to fluorescence of the Evans blue dye used as a counterstain.

that induces strong cellular immunity will be particularly important in this regard, as it has been shown that natural reinfection with RSV does not boost virus-specific T-cell immunity (11). This feature of RSV biology may underlie its ability to reinfect throughout life. Importantly, recurrent URT infection with RSV is the predominant risk factor for bacterial OM. In the studies presented here, we have developed two rodent models of URT infection by RSV to aid in furthering understanding of RSV-induced upper airway disease as well as perhaps future utility in defining the pathogenic mechanisms of RSV predisposition to bacterial OM. Ultimately, both models will be useful to support endeavors to develop a vaccine candidate for the prevention of RSV infection of the upper airway.

ACKNOWLEDGMENTS

Studies conducted in the chinchilla host were supported by discretionary funds to L.O.B. and, in part, by a grant from the NIH/NIDCD, R01 DC05847. Mouse studies were supported by a grant from the NIH, AI47226, to J.E.D.

We thank Jennifer Neelans for manuscript preparation and G. Scott Giebink (in memoriam) for many helpful discussions and encouragement. The Mouse Phenotyping Service of The Ohio State University Department of Veterinary Biosciences provided assistance with immunostaining and data interpretation.

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