Children with acute respiratory syncytial virus (RSV) bronchiolitis often develop sequelae of recurrent wheezing and asthma. To determine whether RSV persists within the lung after resolution of acute bronchiolitis, we examined the lungs of guinea pigs 60 days after intranasal inoculation with either human RSV (n=10) or uninfected cell culture supernatant (n=11). Evidence of viral persistence within the lung was determined by viral culture to test for replicating virus, immunohistochemistry to test for viral protein, and the reverse transcriptase-polymerase chain reaction (RT-PCR) to test for viral genomic RNA. Lungs were also examined histologically for evidence of bronchiolar inflammation or increased numbers of mast cells in the airway walls. All viral cultures were negative; however, there was positive immunohistochemical staining of occasional alveolar macrophages in six of ten RSV-inoculated guinea pigs while RT-PCR was positive in seven of ten RSV-inoculated animals. The six guinea pigs with evidence of RSV by immunohistochemistry and RT-PCR showed excess bronchiolar polymorphonuclear cell infiltrates (p<0.005) but no increase in the number of airway wall mast cells. These results show that RSV protein and genomic RNA can persist in the lungs of experimentally inoculated guinea pigs for at least 60 days after infection and that persistence of the virus within alveolar macrophages might contribute to the pathogenesis of chronic bronchiolar inflammation.

(Rest of the text follows...)

Key words: animal model, persistent infection, respiratory syncytial virus

*From the University of British Columbia Pulmonary Research Laboratory, St. Paul’s Hospital, Vancouver, Canada.
†Recipient of a Parker B. Francis Fellowship in Pulmonary Research.
§Recipient of a B.C. Lung Association Scholarship.
¶Recipient of a Canadian Lung Association Fellowship.
Supported by the Medical Research Council of Canada grant No. 7246 and the National Centres of Excellence for Respiratory Health.

Manuscript received January 1, 1994; accepted for publication March 2.

Reprint requests: Dr. Hegele, UBC Pulmonary Research Laboratory, St. Paul’s Hospital, Vancouver, BC, Canada V6Z 1Y6

Perspective of Respiratory Syncytial Virus Genome and Protein After Acute Bronchiolitis in Guinea Pigs

Richard G. Hegele, M.D., Ph.D.; Shizu Hayashi, Ph.D.; Andrew M. Bramley, Ph.D.; and James C. Hogg, M.D., Ph.D.

---

BALT=bronchus associated lymphoid tissue; CPE=cytopathic effect; ETOH=ethyl alcohol; HEp-2=human laryngeal tumor cell; N=nucleocapsid; PAS=periodic acid-Schiff; PCR=polymerase chain reaction; FN=polymorphonuclear; RSV=respiratory syncytial virus; RT-PCR=reverse transcriptase-polymerase chain reaction
to test for evidence of viral genomic RNA within the lung. Histologic examination was performed to determine if persistence of virus was associated with an inflammatory process of Airways\textsuperscript{18} containing increased numbers of mast cells.\textsuperscript{8} Animals were studied 60 days postinoculation because this is about twice the interval RSV has been cultured from immunocompetent patients after acute bronchiolitis,\textsuperscript{12,13} and this time point permitted comparison of mast cell quantification in the airway walls to previous studies of Sendai virus-injected rats.\textsuperscript{8}

**Materials and Methods**

**Species**

Outbred female guinea pigs (Cam Hartley, Charles River, Montreal, Canada) were randomly assigned into RSV-inoculated (n = 10) or control (n = 11) groups. The two groups were housed in separate rooms equipped with air filters and maintained under identical conditions of plastic cages containing corn cob bedding (Bed o’ Cobs, The Andersons, Maumee, Ohio), access to guinea pig chow (Purina), alfalfa hay cubes, water, and 12-hour alternating light-dark cycles. Animals were maintained in accord with standards of the Canadian Council in Animal Care.\textsuperscript{10} Investigators wore surgical gowns, hats, masks, shoes, covers, and gloves during animal handling and used autoclaved instruments and containers.

**Virus**

The long strain of subgroup A human RSV (American Type Culture Collection, Rockville, Md) was propagated on HEp-2 cell monolayers at multiplicities of infection from 0.01 to 0.1\textsuperscript{28} at 34°C in a humidified incubator containing 5 percent CO\textsubscript{2}. Cell culture medium consisted of minimal essential medium (Dulbecco, Grand Island Biological, Grand Island, NY) supplemented with 5 percent fetal bovine serum (Gibco), 0.292 mg/mL L-glutamine (Gibco), and vitamins (Gibco). No antibiotics or antifungal agents were present in the cell culture medium.

Working stocks of RSV for inoculation of guinea pigs were prepared by adding autoclaved 3-mm diameter glass beads to infected human laryngeal tumor cell (HEp-2) monolayers and by placing the flask on a high speed vortex for 10 s. The lysed suspension was centrifuged at 8000×g at 4°C for 4 min and the resulting supernatant was transferred to a sterile tube for inoculation into guinea pigs. Supernatant from disrupted uninfected HEp-2 cells was obtained in a similar manner for inoculation into control animals.

**Inoculation Procedure**

Guinea pigs were anesthetized via inhalation of 3 to 5 percent halothane (Ayerst Laboratories, Montreal, Canada) and received either 100 μL of RSV-containing supernatant (corresponding to 3.9 ± 0.1·10\textsuperscript{8} plaque-forming units [mean ± SD] by plaque assay on HEp-2 cells) or 100 μL uninfected supernatant by intranasal instillation. Animals were weighed on the day of inoculation, initial body weight, and 60 days postinoculation, final body weight.

**Lung Tissue Processing**

Guinea pigs were anesthetized by intraperitoneal injection of pentobarbital (MTP Pharmaceuticals, Cambridge, Ontario, Canada) and killed by exsanguination. The chest was opened and the heart and lungs were removed en bloc. The right mainstem bronchus was ligated and the right lung was isolated and weighed in a sterile vessel (lung wet weight). The right middle lobe was dissected and processed for viral culture. After transpleural injection with 2 ml ornithine carbamoyltransferase compound (Miles, Elkhart, Indiana), the remainder of the right lung was frozen in liquid nitrogen and stored at −70°C until processed for RT-PCR. The left lung was inflated by intratracheal instillation of phosphate-buffered 4 percent paraformaldehyde and fixed overnight at 4°C. Parasagittal slices of fixed lungs were processed for paraffin embedding on an automated tissue processor (Histomatic, Fisher Scientific, Ottawa, Canada). Serial 4 μL sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and toluidine blue stains.

**Viral Culture**

The fresh right middle lobe was minced into fine pieces with an autoclaved razor blade and digested into single cells by immersion in a filtered solution of 0.25 percent trypsin in phosphate buffered saline (Gibco) at 37°C for 90 min. After centrifugation at 8000×g at room temperature for 4 min, the supernatant was removed and replaced with 1 ml uninfected cell culture medium. The resulting suspension was cocultured with HEp-2 cell monolayers (passage number 300-350) growing in 25 ml flasks containing 4 ml cell culture medium at 34°C. Flasks were examined under an inverted microscope for evidence of syncytial formation, the characteristic cytopathic effect (CPE) of RSV. Cells not showing CPE were passaged at weekly intervals for up to 1 month. A culture was reported as negative when no CPE was observed for the month.

**Immunohistochemistry**

A peroxidase-antiperoxidase protocol, using a polyclonal, rabbit anti-RSV primary antibody (Dako B344, Denmark) was performed on 5 μL thick, paraformaldehyde fixed, paraffin-embedded lung sections as previously described.\textsuperscript{18} Briefly, two lung sections per animal were pretreated with 0.1 percent protease, type XIV (Sigma Chemical, St. Louis, Mo; 57°C, 10 min) to disrupt protein crosslinks induced by fixation, with 0.9 percent H\textsubscript{2}O\textsubscript{2} (room temperature, 25 min) to eliminate endogenous peroxidase activity, and with normal swine serum (Dako; room temperature, 30 min) to prevent nonspecific IgG binding. Incubation with primary rabbit anti-RSV antibody (1 to 300 dilution, room temperature, 90 min) was followed by the following: incubation with biotinylated swine antirabbit secondary antibody (Dako; 1 to 300 dilution, room temperature, 45 min), incubation with peroxidase-conjugated streptavidin (Dako, 1 to 600 dilution, room temperature, 45 min); development in AEC solution, 1 drop 3-amino-9-ethylcarbazole (Sigma) and 1 drop 3 percent H\textsubscript{2}O\textsubscript{2} in 3 ml 0.1 mol/L sodium acetate, pH 5.2 (BDH Chemicals), room temperature, 15 min; and finally counterstaining with hematoxylin (Mayer’s).

Positive control slides included lung sections of RSV-inoculated guinea pigs 6 days postinoculation from a previous study\textsuperscript{11} and two human autopsy cases of culture-proven acute RSV bronchiolitis. Negative control slides were incubated in parallel in the absence of primary anti-RSV antibody or with any irrelevant primary antibody.

**RT-PCR**

Organic extraction of total cellular RNA\textsuperscript{21} was performed on frozen lungs that had been pulverized with a mortar and pestle. Five milliliters of solution D (4 mol/L guanidinium isothiocyanate, Sigma), 25 mM sodium citrate, pH 7 (BDH Chemicals), 0.5 percent sarcosyl (Sigma), 0.1 mol/L 2-mercaptoethanol (Sigma), 0.5 mL 2 mol/L sodium acetate, pH 4.1 (BDH Chemicals), 5 ml water-saturated, molecular biology grade phenol (Gibco BRL, Gaithersburg, Md), and 1 ml of a 24 to 1 mixture of chloroform and isooamyl alcohol (BDH Chemicals) were added to the pulverized lung. This mixture was transferred to an autoclaved 30 ml
Table 1—Comparison of Body Weight, Lung Wet Weight and Lung-to-Body Weight Ratios Between RSV-Inoculated and Control Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Initial Body Wt, g*</th>
<th>Final Body Wt, g*</th>
<th>Lung Wet Wt, g*</th>
<th>Lung to Body Wt Ratio, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>10</td>
<td>364 ± 19</td>
<td>650 ± 53</td>
<td>3.12 ± 1.37</td>
<td>0.48 ± 0.22</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>360 ± 20</td>
<td>661 ± 63</td>
<td>2.39 ± 1.54</td>
<td>0.45 ± 0.18</td>
</tr>
<tr>
<td>p value</td>
<td>NS1</td>
<td>NS1</td>
<td>NS1</td>
<td>NS1</td>
<td>NS1</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†NS=p value >0.05 by Student’s t test.

plastic tube, vigorously agitated for 10 s, placed on ice for 15 min, and centrifuged at 10,000×g at 4°C for 20 min. Five hundred microliter aliquots of the RNA-containing aqueous phase were transferred into autoclaved 1.5 mL microtube tubes, and RNA was precipitated in an equal volume of isopropanol at −20°C more than an hour. After centrifugation at 10,000×g at 4°C for 10 min, the supernatant was aspirated and a second round of RNA extraction in 0.3 ml of solution D, then precipitation in isopropanol, centrifugation, and aspiration of supernatant was performed. The RNA pellets were stored in 250 μL 95 percent ethyl alcohol (EtOH) at −70°C. For RT-PCR, RNA pellets were briefly washed in 70 percent EtOH and dissolved in 25 μL distilled water containing 0.1 percent diethylpyrocarbonate (Aldrich Chemical, Milwaukee, Wis) (DEPC-dH2O). Some aliquots were discarded because of extensive degradation of RNA, but at least one aliquot from each animal was suitable for RT-PCR. We have since found that the yield of intact total cellular RNA can be increased by inflating fresh lungs through the trachea with 5 mL solution D, then freezing it in liquid nitrogen.

A 410 nucleotide region of the RSV nucleoprotein (N) gene was chosen as the target sequence for RT-PCR mainly because oligonucleotides defining its amplification lacked significant sequence homology to other viral, rodent, or primate nucleic acid sequences in the database (Genbank). The RT-PCR was performed as described by Shulinder et al with the following modifications. In the 20 μL reverse transcriptase reaction, the oligonucleotide primer 5′ GGAGGCTCTTAGAGGAAAGAA 3′ (University of Calgary, Alberta, Canada; nucleotides 223 to 244 of the RSV N gene mRNA sense strand) and 8 mM MgCl₂ were used. In the 50 μL PCR amplification reaction, the entire 20 μL reverse transcriptase reaction was included, the flanking primers were 5′ GCTATGCTTTGCTAGTAAAACTC 3′ (University of Calgary; the complement of nucleotides 632 to 690 of the RSV N gene mRNA sense strand), and the oligonucleotide used for reverse transcription and 5 mM MgCl₂ was used. Thirty-five PCR cycles, consisting of denaturation at 94°C for 1 min (5 min during cycle 1) and combined annealing-extension at 70°C for 2 min (10 min 10 min drying cycle 35), were performed on a PCR system (GeneAmp, Perkin Elmer Cetus, Norwalk, Conn.).

The PCR products were run on 1.5 percent agarose gels stained with 0.1 percent ethidium bromide, transferred and fixed onto N membranes (Hybond, Amersham International, Amersham, United Kingdom) by Southern blotting, and hybridized at 65°C for 18 hours with a 32P-labeled oligonucleotide probe 5′ TAGGCTCCAGAATTACGACGTC 3′ (University of Calgary; nucleotides 449 to 473 of the RSV N gene mRNA sense strand; specific activity=3.5·10⁷ counts per minute/μg) in a hybridization solution consisting of 6X SSC (20X SSC is 3 mol/L NaCl, BDH Chemicals; 0.3 mol/L Na citrate-2H₂O, pH 7), 5X Denhardt’s solution (100 X Denhardt’s solution is 2 percent Ficoll 400, Sigma; 2 percent polyvinylpyrrolidone, Sigma; and 2 percent BSA Fraction V, Sigma), 0.5 percent SDS (Fisher Scientific), 0.05 mol/L sodium phosphate buffer, pH 6.8 (BDH Chemicals), and 20 μg/mL Escherichia coli tRNA (Boehringer Mannheim). Posthybridization washes included three 5-minute washes in 6X SSC at room temperature and, to increase stringency, a 60-min wash in 6X SSC at 65°C. For autoradiography, membranes were covered with Saran wrap, exposed to infrared diagnostic x-ray film (Kodak EktaScan IR, Eastman Kodak, Rochester, NY) inside a film cassette (Kodak X-OMatic) with regular intensifying screens, exposed at −70°C from 4 to 72 hours and developed in a film processor (Kodak RP X-Omat Processor).

Positive control samples included RT-PCR of total cellular RNA from RSV-infected HEP-2 cells and PCR of pBR322 plasmid DNA containing the full-length human RSV N gene (gift of Dr. G.W. Wertz, University of Alabama at Birmingham). Negative control samples included RT-PCR of blank samples that did not contain a nucleic acid template and total cellular RNA from uninfected HEP-2 cells. Finally, to test whether RSV may have integrated into the guinea pig’s genomic DNA to produce a latent infection,23 PCR was performed on lung DNA extracted from two RSV-inoculated guinea pigs.

Light Microscopy

The membranous, muscular, noncartilaginous bronchioles were evaluated for epithelial necrosis, mononuclear cell infiltrates, polymorphonuclear (PMN) infiltrates, airway wall edema, hyperplasia of bronchus-associated lymphoid tissue (BALT), and goblet cell metaplasia using a previously described method.18 Slides were coded so the origin of a given section, i.e., RSV-inoculated or control animal, was not known to the microscopist. For a given airway, each parameter was scored from zero (normal) to two (moderate to severe changes) by comparison to standard photomicrographs of guinea pig airways. Ten membranous bronchioles throughout the lung were examined per animal and the observed score for each parameter was expressed as the sum of individual airway scores with a maximum score of 2X10=20 per parameter per animal. A given airway was scored only once per animal and adjacent cuts of previously scored airways were avoided.

Mast Cell Quantification in Bronchioles

Mast cells within bronchiolar walls were counted using a minor modification of the method of Castleman et al.24 Briefly, 30 high-power fields (400 X magnification) of toluidine blue-stained lung sections from paraformaldehyde-fixed, paraffin embedded blocks from each animal were viewed at sequential 0.16 mm intervals. All mast cells between the epithelial and smooth muscle layers of the airway wall were counted and expressed as the mean ± SD of mast cells/mm wall.

Statistical Analyses

Statistical analyses were performed using a statistical software (Systat Version 5.1, Systat, Evanston, Ill) for the 10 RSV-inoculated and 11 control guinea pigs. The Student’s t test was used to compare mean body weight, lung wet weights, lung-to-body weight ratios, and number of airway wall mast cells per mm wall between the RSV-inoculated and control groups. A p value <0.05 was considered to be statistically significant. The Mann
Table 2—Documentation of RSV Within the Lungs of Guinea Pigs

<table>
<thead>
<tr>
<th>Technique</th>
<th>RSV-Inoculated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>0/10</td>
<td>0/11</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>6/10</td>
<td>0/11</td>
</tr>
<tr>
<td>RT-PCR*</td>
<td>7/10</td>
<td>0/11</td>
</tr>
</tbody>
</table>

*All animals positive for RSV protein by immunohistochemistry were also positive for RSV genomic RNA by RT-PCR.*

Whitney U test was used to compare scores (ordinal variables) for each of the six histologic features between the RSV-inoculated and control groups; to account for multiple comparisons, a sequential rejecting Bonferroni procedure was used to evaluate statistical significance at sequential p value $<$0.0083 (0.05/6), $<$0.01 (0.05/5), etc, until all six features had been analyzed. Results of viral culture, immunohistochemistry, and RT-PCR are reported as either positive (unequivocal signal observed) or negative (no signal observed).

RESULTS

Table 1 shows the results of initial and final body weights, lung wet weights, and lung-to-body weight ratios between the RSV-inoculated and control groups. There were no statistically significant differences between the RSV-inoculated and control groups.

Table 2 shows the results of viral culture, immunohistochemistry, and RT-PCR. Although no replicating viruses were isolated by culture, lung sections from six of ten RSV-inoculated guinea pigs showed intracytoplasmic staining for RSV protein in occasional cells within alveolar spaces (Fig 1a) with morphologic features of alveolar macrophages, round cells measuring 20 to 30 μm diameter, with a reniform nucleus. The lack of cross-reactivity between human and guinea pig macrophage epitopes for available antimacrophage antibodies precluded further immunocytochemical characterization of these alveolar macrophage-like cells. No immunostaining was observed in lung sections from uninfected control guinea pigs or in sections from RSV-inoculated animals incubated in the absence of primary antibody (Fig 1b).

Figure 2 shows the results of agarose gel electrophoresis and autoradiography for RT-PCR. A 410 base pair band corresponding to the predicted PCR product was present by autoradiography in seven of ten RSV inoculated animals. The RT-PCR was negative in lung from uninfected control guinea pigs, in blank control samples, and in total cellular RNA from uninfected HEP-2 cells. Furthermore, there was no evidence of viral integration into the guinea pig genome to produce latent infection because no bands were detected after PCR of lung DNA from two animals positive for RSV by RT-PCR (Table 2).

All animals with immunohistochemical evidence of RSV protein also had intrapulmonary viral genomic RNA shown in the contralateral lung by RT-PCR (Table 2). One RSV-inoculated animal, however, showed evidence of viral genomic RNA by RT-PCR but no immunohistochemical evidence of RSV protein in the contralateral lung.

Figure 3 shows that the subgroup of six RSV-inoculated guinea pigs with positive RSV immunohistochemistry, and RT-PCR had statistically significant bronchiolar PMN infiltrates ($p<0.005$) compared with the control group. There were no statistically significant differences in the number of airway wall mast cells per millimeter wall, even when all ten RSV-inoculated guinea pigs (2.1 ± 1.7, mean ± SD) or the subgroup of six animals with positive RSV immunohistochemistry and RT-PCR (1.8 ± 0.6) were compared with the control group (1.7 ± 0.8).

DISCUSSION

These studies show that human RSV genomic RNA and protein are present within the lungs for at least 60 days after intranasal inoculation of about 4,000 plaque-forming units into anesthetized, juvenile guinea pigs. Furthermore, the presence of viral genomic RNA and protein is associated with excess bronchiolar PMN infiltrates ($p<0.005$) without differences in mean body weight, lung wet weight, lung-to-body weight ratio, or number of mast cells.
per unit length of airway wall.

The identification of intrapulmonary viral genomic RNA by RT-PCR suggests that RSV remained viable over 60 days because RNA from inactivated viruses would likely have been digested by endogenous ribonuclease. This finding is in contrast to previous observations of influenza virus-infected mice, where chronic persistence of viral protein within the lung has been documented without evidence of viral genome. In this instance, the persistent influenza viral antigens in the lung could have represented residual protein from inactivated viruses.

In the current studies, replicating viruses were not cultured from lung parenchymal digests and, therefore, persistent RSV infection was not definitely established. Negative cultures, however, may have been related to sampling error. RSV remaining cell-associated within alveolar macrophages or RSV persisting in a nonreplicative form. Continuous replication of RSV, however, may not be necessary for alteration of cellular function; for example, heat-inactivated RSV is comparable with live, replicating virus in stimulating alveolar macrophages to secrete the inflammatory cytokines, interleukin-6 and -8, in vitro. Finally, latent infection produced by viral integration into host genomic DNA was a less likely mechanism for negative cultures, because there was no PCR evidence of the target RSV N gene sequence in DNA extracted from lungs of guinea pigs where the RNA was positive by RT-PCR.

The observation of excess bronchiolar PMN infiltrates limited to the subgroup of guinea pigs with positive viral immunohistochemistry and RT-PCR is consistent with a direct and an indirect role for RSV in the pathogenesis of chronic airway inflammation. For example, RSV could induce alveolar macrophages to secrete interleukin-8, a neutrophil chemoattractant, by a novel mechanism in which inflammatory mediators secreted into the airspace stimulate recruitment of inflammatory cells to the airways. Alternatively, the immunohistochemistry results do not exclude the possibility of persistent, low-level RSV infection of airway epithelial cells in which there is sufficient virus to stimulate ongoing airway inflammation but an insufficient amount to stain with anti-RSV antibody. In this situation, the positive-staining alveolar macrophages could be acting as scavengers for free virus released by these other cell types.

Another possibility is that chronic airway inflammation may be induced indirectly through virus-induced alterations of alveolar macrophage function. For example, viral infections impair the ability of alveolar macrophages to clear inhaled substances from the peripheral lung and prolonged exposures to allergens might facilitate antigen sensitization and induction of atopy. Viral infections can also decrease the phagocytic and bactericidal capacity of alveolar macrophages such that airway inflammation could result from bacterial superinfections. Despite precautions to prevent undesired infections of guinea pigs, the possibility of bacterial superinfection cannot be entirely excluded because "specific pathogen-free" guinea pigs were not used in the current studies.

**Figure 2.** Results of agarose gel electrophoresis (top portion) and autoradiography (bottom portion) after RT-PCR for a 410 base pair sequence of the human RSV nucleocapsid gene. Lanes are identified by the starting material of each sample. Figure 2a shows lanes 1 and 16: PUC 18-Hinf I digested size markers (base pair). Lanes 2 to 5 and 11 to 13 are total cellular RNA from lungs of uninfected control guinea pigs; lanes 6-10, 14, 15 are total cellular RNA from lungs of RSV-inoculated guinea pigs. The autoradiograph shows a 410 base pair RT-PCR product in four of seven RSV-inoculated animals (Lanes 6, 9, 10, 14) indicative of intrapulmonary RSV genomic RNA and no bands in seven of seven uninfected control animals. Figure 2b shows lane 1 is PUC 18-Hinf I digested size markers (base pair). Lanes 2 to 5 are the total cellular RNA from lungs of uninfected control animals; lanes 6 to 8 are the total cellular RNA from lungs of RSV-inoculated guinea pigs. Lane 9 is the total cellular RNA from RSV-infected HEp-2 cells; lanes 10 and 11 are DNA extracted from the lungs of the same guinea pigs as RNA shown in Lanes 6 and 8 of Figure 2b; lane 12 is the blank sample; lane 13 is the RSV N gene plasmid DNA. Lane 14 is the total cellular RNA from uninfected HEp-2 cells undergoing RT-PCR. The autoradiograph shows a 410 base pair RT-PCR product in three of three RSV-inoculated animals (Lanes 6, 7, 8) indicative of intrapulmonary RSV genomic RNA and in known sources of the RT-PCR target sequence (Lanes 9, 13). No bands are present in four of four uninfected control animals, total DNA from two guinea pigs with positive RT-PCR, in a blank negative control sample and in uninfected HEp-2 cell total RNA.
Quantitative interpretations of immunohistochemistry and RT-PCR results must be guarded because of technical considerations. For example, no false-positive immunostaining for RSV protein was observed in paraformaldehyde-fixed, paraffin-embedded control specimens, and this high specificity was achieved by an uncalculated loss of sensitivity. Regarding quantitative RT-PCR, competitive amplification of known amounts of the target nucleic acid sequence is a reliable strategy that requires more intact RNA than was available in the current studies. Quantitative interpretations of RT-PCR autoradiographs in these studies are further complicated by the presence of a minor band that probably represented virus-specific, single-stranded DNA PCR products. The possibility of false-negative results by RT-PCR can be discounted because the protocol used in these studies has successfully amplified as few as five copies of the target sequence in serial dilutions of RSV N gene plasmid cDNA (data not shown). Despite the immunohistochemistry and RT-PCR results being limited to qualitative interpretations, they clearly show that RSV protein and genomic RNA persisted within the lung far longer than previously reported in studies that used viral culture as the standard for documenting evidence of RSV within the lung.

In contrast to the observations of Sendai virus-infected rats, RSV-infection of guinea pigs did not induce increased numbers of mast cells in the bronchial walls 60 days postinoculation. This apparent discrepancy might reflect species or age-related factors in the mast cell content of airway walls because, in aldehyde-fixed tissues stained with toluidine blue, the uninfected control guinea pigs reported herein had about twice the number of mast cells per millimeter in their airways compared with control rats, but only one-half the number reported in 400 to 500 g guinea pigs (Dunkin-Hartley). Alternatively, factors related to the species tropism of viruses or viral-host interactions might be involved since rats spontaneously develop respiratory tract infections to Sendai virus while guinea pigs are not natural hosts to human RSV. Moreover, it remains unclear whether the numbers of mast cells in the airway walls are related to the pathogenesis of airway hyperresponsiveness in patients with asthma.

In summary, these experiments have established that intranasal inoculation of human RSV into guinea pigs results in persistence of viral protein and genomic RNA within the lung for at least 60 days, a longer interval than previously reported. Second, these experiments have provided evidence that persistence of viral protein and genome is associated with significant bronchial PMN infiltrates. We speculate that RSV can induce functional alterations of alveolar macrophages that might contribute to the pathogenesis of chronic airway inflammation. Experimental human RSV infection of the guinea pig lung provides a model that may be useful in investigating the mechanisms of postbronchiolitis wheezing and asthma.

FIGURE 3. Airway histologic scores of guinea pigs on day 60 post-inoculation. The subgroup of 6 guinea pigs with positive RSV immunohistochemistry and RT-PCR (n=6) shows statistically significant bronchial PMN infiltrates (p<0.005) compared with the uninoculated control group (n=11).
ACKNOWLEDGMENTS: The authors thank Margaret McLean, Lynne Carter, Diane Minshall, Shaun Grannlee, and Simon Bicknell for technical assistance, Stuart Greene for photography, Lorri Verburgt for statistical analyses, and Maureen Brooks for typing the manuscript.

REFERENCES

3. Pullan CR, Hey EN. Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. BMJ 1982; 284:1665-69