Respiratory Syncytial Virus Persistence in the Lungs Correlates with Airway Hyperreactivity in the Mouse Model

Dora Estripeaut,1,a Juan Pablo Torres,1,a Cynthia S. Somers,1 Claudia Tagliabue,1 Shama Khokhar,1 Vijay G. Bhoj,2 Steve M. Grube,1 Aneta Wozniakowski,1 Ana M. Gomez,2 Octavio Ramilo,1 Hasan S. Jafri,1,b and Asuncion Mejias1

1Department of Pediatrics, Division of Pediatric Infectious Diseases, 2Department of Molecular Biology, and 3Department of Pathology, University of Texas Southwestern Medical Center and Children’s Medical Center Dallas, Texas

Background. Previous studies in mice showed that respiratory syncytial virus (RSV) infection was associated with RSV RNA persistence. This study was designed to characterize the significance of RSV RNA persistence and its relation to RSV-induced chronic airway disease.

Methods. Mice were inoculated with live RSV, UV light–treated RSV, heat-inactivated RSV, or medium. Bronchoalveolar lavage fluid samples were obtained and lung specimens were harvested on days 1, 5, and 42 after inoculation to assess lung inflammation, lung mRNA expression of interleukin (IL)–4, IL-5, IL-15, and interferon (IFN)–γ; RSV loads were assessed by culture and real-time polymerase chain reaction (PCR) and correlated with pulmonary function.

Results. During the acute phase of infection, RSV loads as indicated by culture and PCR were significantly higher in mice inoculated with live RSV. On day 42, RSV RNA remained detectable only in mice inoculated with live or UV light–treated RSV. Lung inflammation, IFN-γ:IL-4 mRNA expression ratios, airway obstruction (AO), and airway hyperreactivity (AHR) were significantly increased in mice inoculated with live RSV. AO on day 5 and AHR on day 42 were significantly correlated with RSV RNA copy number in lung samples.

Conclusions. Infection with live RSV induced acute and chronic airway disease that was associated with a predominantly Th-1 immune response and RSV RNA persistence that significantly correlated with pulmonary function abnormalities.

Respiratory syncytial virus (RSV) infection is the leading cause of hospitalization in infants and young children worldwide [1, 2]. In addition to the acute morbidity [3], the association between RSV lower respiratory tract infection and the development of recurrent wheezing has been clearly established in several well-controlled prospective studies [4–7]. In fact, among children hospitalized for RSV bronchiolitis, studies indicate that >30% will develop persistent wheezing until 13 years of age, which may extend into early adulthood [4, 5]. The nature of this association is not well understood, and both the host and the virus likely contribute to the development of RSV-induced long-term airway disease [8, 9].

Recent evidence derived from in vitro experiments [10], animal models [11–14], and studies in adults [15] suggests that RSV may persist “latently” or at a low level of viral replication in immunologically privileged sites in the lung, avoiding immune detection and elimination [16]. However, questions have arisen regarding the significance, if any, of RSV persistence.

This study was designed to characterize the significance of RSV RNA persistence in the lungs by using a mouse model of RSV infection. To this end, we determined whether the establishment of RSV RNA persistence in the lungs required active viral replication and whether RSV RNA persistence correlated with the development of RSV-induced chronic pulmonary disease.

Received 10 March 2008; accepted 29 May 2008; electronically published 1 October 2008.

Potential conflicts of interest: none reported.


Financial support: RGK Foundation (grant to A.M.); National Institutes of Health (grant 1 U11 RR024962–01 to A.M.); American Lung Association (grant to H.S.J.).

a D.E. and J.P.T. contributed equally to this work.

b Present affiliation: Medimmune, Gaithersburg, Maryland.

Reprints or correspondence: Dr. Asuncion Mejias, Department of Pediatrics, Division of Pediatric Infectious Diseases, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390–9093 (Asuncion.Mejias@UTSouthwestern.edu).

The Journal of Infectious Diseases 2008; 198:1435–43
© 2008 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2008/19810-0004$15.00
DOI: 10.1086/592714
MATERIALS AND METHODS

Animals. BALB/c female mice (7–8 weeks old) were purchased from Charles River Laboratories. The animals were housed in the animal care facility in separate filter-top cages, in accordance with the study design. Their pathogen-free status was confirmed by use of sentinel mice that were regularly tested for different pathogens, as described elsewhere [17–19]. This study was approved by the Institutional Animal Care and Research Advisory Committee at the University of Texas Southern Medical Center at Dallas.

Virus. Human RSV stock A2 was propagated on HEp-2 cells and stored, as described elsewhere [17, 18]. HEp-2 cells were maintained in Eagle’s minimal essential medium (EMEM) supplemented with HEPES, glutamine, streptomycin, penicillin G, and 10% fetal bovine serum. RSV A2 was treated with UV light irradiation for 60 min, using a UV cross-linker (Fisher Biotech) [20], or inactivated by heat at 60°C over 30 min [20–22]. Viral titers were determined by plaque assay [17, 23] and real-time polymerase chain reaction (PCR), with lower limits of detection of 1.7 log_{10} pfu/mL and 10 copies/mL, respectively [12]. The viruses used for each individual experiment were tested in triplicate at the time of inoculation.

RSV inoculation. Mice were intranasally inoculated with 10^{7.3–10} pfu/mL (10^{8.8–10} log_{10} RSV RNA copies/mL) of live RSV, UV light–treated RSV, heat-inactivated RSV (HI-RSV), and 10% fetal bovine serum. RSV A2 was treated with UV light irradiation for 60 min, using a UV cross-linker (Fisher Biotech) [20], or inactivated by heat at 60°C over 30 min [20–22]. Viral titers were determined by plaque assay [17, 23] and real-time polymerase chain reaction (PCR), with lower limits of detection of 1.7 log_{10} pfu/mL and 10 copies/mL, respectively [12]. The viruses used for each individual experiment were tested in triplicate at the time of inoculation.

Virus. Human RSV stock A2 was propagated on HEp-2 cells and stored, as described elsewhere [17, 18]. HEp-2 cells were maintained in Eagle’s minimal essential medium (EMEM) supplemented with HEPES, glutamine, streptomycin, penicillin G, and 10% fetal bovine serum. RSV A2 was treated with UV light irradiation for 60 min, using a UV cross-linker (Fisher Biotech) [20], or inactivated by heat at 60°C over 30 min [20–22]. Viral titers were determined by plaque assay [17, 23] and real-time polymerase chain reaction (PCR), with lower limits of detection of 1.7 log_{10} pfu/mL and 10 copies/mL, respectively [12]. The viruses used for each individual experiment were tested in triplicate at the time of inoculation.

Histopathology. Formalin-fixed lungs were paraffin embedded, sectioned, and stained with hematoxylin-eosin prior to light microscopic examination, as described elsewhere [12, 17, 18]. The histopathologic score (HPS) was determined by a pathologist unaware of the infection status of the animals. This HPS system assigns values from 0 (no inflammation) to 21 (severe inflammation) and is based on the grading of 5 parameters: (1) peribronchiolar and bronchial infiltrates, (2) bronchiolar and bronchial luminal exudates, (3) perivascular infiltrates, (4) parenchymal monocytic infiltrate, and (5) parenchymal pneumonia (granulocytic infiltrates in the alveolar spaces). This scoring system has been validated elsewhere in the RSV mouse model [17, 18, 26].

RSV quantitative culture and real-time PCR. Eighty percent confluent 2-day-old HEp-2 cells were used to assess viral loads in BAL fluid samples, as described elsewhere [17, 18]. The remaining supernatant was frozen at −80°C for viral load measurement by real-time PCR. One sample per mouse was evaluated as a single specimen. In brief, RNA from BAL fluid and whole-lung specimens was extracted by using ion exchange minicolumns (Qiagen RNeasy Mini Kit), in accordance with the manufacturer’s instructions. cDNA was prepared by reverse transcription using 2.5 μmol/L random hexamers for 10 min at 22°C, 30 min at 42°C, and 5 min at 95°C. Real-time PCR was performed by using an Applied Biosystems ABI-7300 HT sequence detector with 10 μL of cDNA in a total volume of 50 μL of master mix and the following run conditions: 1 cycle for 2 min at 50°C and 10 min at 95°C, followed by 50 cycles for 15 sec at 95°C and 60 sec at 60°C. Known concentrations of RSV A2 were used to derive a standard curve. Standards and negative controls
were run together with each PCR assay. Quantitative real-time PCR was used, targeting the conserved region of the RSV N gene. Forward (5'-AGA TCA ACT TCT GTC ATC CAG CAA) and reverse (5'-TTC TGC ACA TCA TAA TTA GGA GTA TCA AT) primers amplified an 85-bp region containing the 25-mer FAM-labeled probe (5'-CAC CAT CCA ACG GAG CAC AGG AGA T), as described elsewhere [27].

Cytokine expression in whole-lung specimens as measured by semiquantitative PCR. Quantification of IL-15 (Mm00434210_m1), IFN-γ (Mm00801778_m1), IL-4 (Mm00445259_m1), and IL-5 (Mm00434210_m1) mRNA (ABI) was performed by real-time PCR with an ABI-PE Prism 7300 HT sequence detection system, as described elsewhere [12, 28]. In brief, 20-μL reaction mix was prepared with 2 μL of cDNA, 10 μL of master mix, 7 μL of DNase/RNase water, and 1 μL of assay on demand containing forward and reverse primers and a fluorescent TaqMan probe, designed and optimized for the genes of interest. Samples (8–10 per time point for each group) were tested in duplicate. The PCR threshold cycle (C_T) for each target product was set in relation to the amplification of the housekeeping gene, β-actin. The C_T value was defined as the number of PCR cycles required for the fluorescent signal to exceed the detection threshold value. The relative mRNA expression of IL-15, IFN-γ, IL-4, and IL-5 was calculated by using the comparative ΔΔC_T method, described elsewhere [28, 29]. Briefly, the relative quantitation of the target value, normalized to the β-actin gene and relative to a calibrator (controls), is expressed as 2^-ΔΔC_T (n-fold induction compared with the control animals).

Statistical methods. The differences between groups were tested by using the 1-way analysis of variance (ANOVA) or Kruskal-Wallis 1-way ANOVA, according to data distribution. If this test demonstrated a significant difference between groups (P < .05), Dunn’s test to correct for multiple comparisons was used. The Spearman rank order test was used for correlations. For all statistical analyses, SigmaStat software (version 3.0; SPSS Science) was used.

RESULTS

RSV Loads Measured by Quantitative Plaque Assay in Mice Inoculated with Live, UV Light-Treated, or HI-RSV

As already shown in this model, viral loads in BAL fluid samples from mice infected with live RSV increased significantly from day 1 to day 5 after inoculation (P < .001), demonstrating active viral replication. On days 1 and 5, BAL fluid RSV loads were significantly higher in mice inoculated with live RSV than in all other groups (P < .001) (figure 1A). To a lesser degree, mice inoculated with UV light–treated RSV—which was partially attenuated but not inactivated—had viable virus recovered in BAL fluid and a significant increase in BAL fluid viral loads from day 1 to day 5 (P = .004). Similarly, in mice inoculated with different concentrations of live RSV, BAL fluid RSV loads were directly proportional to the inoculum concentration (2.7 ± 0.41 pfu/mL for the lower inoculum vs. 3.4 ± 0.33 pfu/mL for the higher inoculum; P = .009). In HI-RSV and noninfected controls, BAL fluid RSV loads were reduced to below the limit of detection of the assay (figure 1A). On day 42 after inoculation, no virus was detected by plaque assay in any of the study groups.

RSV Persistence Demonstrated with Real-Time PCR

Real-time PCR detected both viable and inactivated virus during the acute phase of the disease and demonstrated that live RSV is required to establish RSV RNA persistence.

BAL fluid samples. On day 1, the number of RSV RNA copies in BAL fluid was comparable among mice inoculated with live RSV, UV light–treated RSV, or HI-RSV, likely reflecting the RSV RNA introduced with the viral inoculum. On day 5, however, RSV RNA loads were significantly higher in mice inoculated with live RSV, compared with both the UV light–treated RSV group and the HI-RSV group (figure 1B). Indeed, mice inoculated with HI-RSV showed a significant decline in the number of RSV RNA copies from day 1 to day 5 (P < .001), further suggesting that the RSV RNA measured during this acute phase reflects the viral inoculum that is subsequently degraded over time. Forty-two days after inoculation, RSV RNA was not detected in BAL fluid samples from any group. Noninfected controls had no RSV RNA detected throughout the experiments.

Whole-lung samples. On days 1 and 5 after inoculation, whole-lung RSV RNA loads were significantly higher in mice inoculated with live RSV, compared with all other groups (figure 1C). On day 42, RSV RNA remained detectable in mice infected with live RSV and, to a lesser degree, in the UV light–treated RSV group. As expected, RSV RNA copy number was significantly higher in mice inoculated with live RSV than in mice inoculated with attenuated UV light–treated RSV. Uninfected control animals and those inoculated with HI-RSV did not have RSV RNA detected at this later point (figure 1C). Mice inoculated with different concentrations of live RSV showed similar lung RSV RNA loads on day 5 (7.2 ± 0.05 log_{10} RNA copies/mL for the lower vs. 7.3 ± 0.02 log_{10} RNA copies/mL for the higher inoculum; P > .05) and day 42 after inoculation (6.53 ± 0.005 log_{10} RNA copies/mL for the lower vs. 6.49 ± 0.04 log_{10} RNA copies/mL for the higher RSV inoculum; P > .05). Taken together, these results indicate that inoculation with active replicating RSV is required to establish RSV RNA persistence.

Viable RSV and Induction of Acute and Chronic Lung Inflammation

Compared with mice inoculated with HI-RSV and noninfected control animals, mice infected with live RSV showed significantly higher HPS results, with a dense inflammatory infiltrate...
and severe pneumonia that peaked on day 5 after inoculation ($P < .001$). Mice inoculated with attenuated UV light–treated RSV showed less inflammation but similar histologic findings, compared with mice inoculated with live RSV (mean HPS ± SD, 8.9 ± 3.4 in mice inoculated with UV light–treated RSV vs. 11.3 ± 2.2 in mice inoculated with live RSV; $P = .09$) (figure 2). Although the acute inflammatory infiltrates gradually declined and changed over time, they persisted in mice inoculated with either live or UV light–treated attenuated RSV. On day 42, lung HPS values in mice inoculated with live or attenuated virus were significantly higher than those in mice inoculated with HI-RSV and uninfected control animals ($P < .001$). On both days 5
and 42, mice inoculated with HI-RSV or inoculated with medium showed no signs of lung inflammation (HPS, 0) (figure 2). Because whole-lung specimens were used either to measure RSV RNA loads or to assess lung inflammation, we could not compute direct correlations between lung RNA loads and HPS.

**Real-Time PCR Measurement of IL-4, IL-5, IL-15, and IFN-γ mRNA Expression in the Lung**

Semiquantitative real-time PCR measurement of IL-4, IL-5, IL-15, and IFN-γ mRNA expression in the lung demonstrated a predominantly Th-1 immune response. Relative expression of IL-4 and IL-5 mRNA in the lung were detected at low levels and their median values were comparable among all groups on days 1, 5, and 42 after inoculation (table 1). On day 1, the relative expression of IL-15 mRNA was significantly higher in mice inoculated with live or UV light–treated RSV than in those inoculated with HI-RSV (table 1). IFN-γ mRNA relative expression and IFN-γ/IL-4 ratios were significantly and persistently higher only in mice inoculated with live RSV at all time points evaluated (figure 3).

**Pulmonary Function Abnormalities**

Inoculation with live RSV, but not with inactivated virus, was associated with acute and long-term pulmonary function abnormalities, which correlated with RSV RNA loads

**RSV infection and AO.** In the acute phase of the disease, mice infected with live RSV, compared with the other study groups, developed significant AO, as defined by increased spontaneous Penh values, with 2 peaks on days 1 and 5. From days 4 to 7, mice inoculated with the attenuated UV light–treated RSV also developed some degree of AO, but significantly less than mice inoculated with live RSV (P < .001). Mice inoculated with the high and low RSV inocula developed AO in a dose-responsive manner (Penh on day 5, 1.34 ± 0.1 vs. 0.87 ± 0.3, respectively; P = .04). Penh values in mice inoculated with either HI-RSV or medium were similar, with no evidence of AO at any time point (figure 4A). After day 5, AO gradually declined in the live and UV light–treated RSV groups, and Penh returned to baseline values by day 11. The AO peak on day 5 correlated with the worst HPS (P = .02; r = 0.72), BAL fluid RSV RNA loads (P < .001; r = 0.76), and RSV RNA loads in the lung (P < .001; r = 0.7) in mice infected with live RSV (figure 4B).

**RSV infection and AHR.** Aerosolized methacholine elicited significantly increased AHR in mice infected with live RSV, compared with all other groups. At 5 and 6 weeks after inoculation, mice infected with live RSV had significantly greater AHR than mice infected with HI-RSV and uninfected controls. Mice inoculated with the attenuated UV light–treated RSV showed a trend toward less AHR than mice infected with live RSV, but at 5 weeks after inoculation they had significantly higher AHR than mice infected with HI-RSV (figure 5A). Mice inoculated with different doses of the same RSV stock did not show significant differences in AHR (ΔPenh on day 42, 1.33 ± 0.2 for the high vs. 1.47 ± 0.4 for the low inoculum; P > .05).

---

**Figure 2.** Lung inflammation assessed in the different experimental groups. Photomicrographs (magnification, ×100) of hematoxylin-eosin–stained lung tissue obtained at 5 (A–D) and 42 (E–H) days after inoculation from mice inoculated with live RSV (A and E), UV light–treated RSV (B and F), heat-inactivated RSV (HI-RSV) (C and G), or noninfected control mice (D and H). Five days after inoculation, tissue from mice inoculated with live RSV (A) showed a mononuclear perivascular infiltrate and an extensive alveolar infiltrate composed of monocytes and/or macrophages and neutrophils, whereas mice inoculated with UV light–treated RSV (B) showed less severe inflammation, with milder perivascular mononuclear infiltrates and only scant alveolar inflammation. In contrast, mice inoculated with HI-RSV (C) or inoculated with medium (D) showed no evidence of lung inflammation. At day 42, mice inoculated with live RSV (E) exhibited some thick circumferential perivascular infiltrates composed of lymphocytes and plasma cells, whereas mice inoculated with UV light–treated RSV (F) demonstrated only focal noncircumferential perivascular mononuclear infiltrates. Mice inoculated with HI-RSV (G) or inoculated with medium (H) did not show any perivascular infiltrates.
To determine whether there was any relationship between RSV persistence and the long-term pulmonary function abnormalities observed after inoculation with live RSV, RSV RNA loads were correlated with AHR (ΔPenh) in the chronic phase. On day 42 after inoculation, the number of RSV RNA copies in the lung was significantly correlated with AHR (P < .01; Table 1).

**Table 1. Cytokine mRNA expression measured by real-time polymerase chain reaction in whole-lung specimens from mice inoculated with sterile medium, heat-inactivated respiratory syncytial virus (HI-RSV), UV light–treated RSV, or live RSV.**

<table>
<thead>
<tr>
<th>Time after inoculation, treatment group</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-15</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1: acute infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.15 (0.69–1.78)</td>
<td>0.99 (0.97–1.02)</td>
<td>1.01 (0.94–1.05)</td>
<td>1.02 (0.79–1.35)</td>
</tr>
<tr>
<td>HI-RSV</td>
<td>0.97 (0.70–1.22)</td>
<td>0.96 (0.73–1.04)</td>
<td>0.77 (0.77–0.92)</td>
<td>0.78 (0.66–1.66)</td>
</tr>
<tr>
<td>UV light–treated RSV</td>
<td>1.17 (0.64–1.65)</td>
<td>0.50 (0.37–1.16)</td>
<td><strong>2.26 (1.45–3.15)</strong></td>
<td>3.79 (2.23–6.49)</td>
</tr>
<tr>
<td>Live RSV</td>
<td>0.68 (0.55–2.16)</td>
<td>0.67 (0.40–0.88)</td>
<td><strong>3.15 (1.48–3.79)</strong></td>
<td><strong>7.02 (3.95–11.66)</strong></td>
</tr>
<tr>
<td><strong>Day 5: acute infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.04 (0.81–1.19)</td>
<td>1.00 (0.86–1.20)</td>
<td>1.00 (0.82–1.27)</td>
<td>1.00 (0.87–1.17)</td>
</tr>
<tr>
<td>HI-RSV</td>
<td>1.31 (0.76–2.40)</td>
<td><strong>0.71 (0.54–1.09)</strong></td>
<td>0.90 (0.84–1.03)</td>
<td>1.23 (1.01–2.10)</td>
</tr>
<tr>
<td>UV light–treated RSV</td>
<td>1.63 (0.53–2.82)</td>
<td>0.38 (0.15–0.74)</td>
<td>0.77 (0.65–0.95)</td>
<td><strong>7.79 (7.95–128.56)</strong></td>
</tr>
<tr>
<td>Live RSV</td>
<td>1.40 (0.66–1.75)</td>
<td>0.14 (0.09–0.45)</td>
<td>0.77 (0.69–0.90)</td>
<td><strong>45.30 (25.90–77.03)</strong></td>
</tr>
<tr>
<td><strong>Day 42: chronic infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.15 (0.69–1.64)</td>
<td>0.96 (0.88–1.21)</td>
<td>1.05 (0.92–1.08)</td>
<td>1.03 (0.80–1.28)</td>
</tr>
<tr>
<td>HI-RSV</td>
<td>0.89 (0.71–1.14)</td>
<td>0.57 (0.42–0.88)</td>
<td>0.68 (0.67–0.80)</td>
<td>1.40 (1.23–1.42)</td>
</tr>
<tr>
<td>UV light–treated RSV</td>
<td>1.46 (1.26–2.19)</td>
<td>0.52 (0.47–0.99)</td>
<td>0.70 (0.63–0.80)</td>
<td><strong>2.33 (2.12–4.55)</strong></td>
</tr>
<tr>
<td>Live RSV</td>
<td>1.14 (1.09–1.28)</td>
<td>0.67 (0.62–0.80)</td>
<td>0.70 (0.67–0.78)</td>
<td><strong>3.55 (2.59–4.45)</strong></td>
</tr>
</tbody>
</table>

**NOTE.** Results are expressed in relative mRNA units (n-fold induction, compared with control animals) calculated as $2^{-\Delta CT}$. Values are medians (25th–75th percentiles) of 8–10 mice per group from 2 independent experiments; all samples were tested in duplicate. Differences between groups of animals were tested using the Kruskal-Wallis test as a nonparametric 1-way analysis of variance; bold type indicates the cytokines that were significantly different by this test. If this test demonstrated a significant difference between groups (P < .05), Dunn’s method of correcting for multiple comparisons was used to evaluate the significance of differences between the different regimens evaluated. Ct, threshold cycle; HI-RSV, heat-inactivated respiratory syncytial virus; IFN, interferon; IL, interleukin.

a P < .001 for live RSV vs. HI-RSV and noninfected controls and for UV light–treated RSV vs. HI-RSV.
b P < .002 for live RSV vs. HI-RSV and noninfected controls.
c P < .04 for HI-RSV vs. live RSV.
d P < .001 for live RSV vs. HI-RSV and controls and for UV light–treated RSV vs. HI-RSV.

To determine whether there was any relationship between RSV persistence and the long-term pulmonary function abnormalities observed after inoculation with live RSV, RSV RNA loads were correlated with AHR (ΔPenh) in the chronic phase. On day 42 after inoculation, the number of RSV RNA copies in the lung was significantly correlated with AHR (P < .01; Figure 3).

**Figure 3.** Semiquantitative real-time polymerase chain reaction measurement of lung interferon (IFN)-γ:interleukin (IL)-4 mRNA ratios. Lung IFN-γ:IL-4 mRNA expression ratios were assessed in 8–10 mice per time point and group in 3 independent experiments on days 1 and 5 (A and B) and on day 42 (C) after inoculation. Mice were intranasally inoculated with live respiratory syncytial virus (RSV), UV light–treated RSV, heat-inactivated RSV, or 10% Eagle’s minimal essential medium. Values are medians and 25th–75th percentiles. The Kruskal-Wallis test was used for comparisons with noninfected control mice; *P < .001. If this test demonstrated a significant difference between groups, Dunn’s test to correct for multiple comparisons was used; differences were considered significant at P < .05.
DISCUSSION

It has been suggested that the persistence of RSV RNA in the lungs could contribute to the development of chronic airway disease.}

\[ r = 0.8 \] (figure 5B). Taken together, these results provide further evidence that inoculation with live RSV is required for both development of RSV-induced chronic airway disease and the establishment of RSV persistence.
disease [12–14, 26]. The presence of low-level viral replication could represent a persistent stimulus responsible for the chronic inflammation and the AHR observed in murine models of RSV infection and in humans [12, 13, 30].

In this study the application of real-time PCR demonstrated that (1) the long-term presence of RSV RNA in the lungs required active viral replication during the acute phase of RSV disease, (2) viral replication and disease severity were directly associated with the degree of inoculum attenuation and/or inactivation, (3) IFN-γ mRNA expression remained elevated during the chronic phase of the disease, and (4) RSV RNA loads correlated not only with acute AO but also with the long-term AHR detected at 6 weeks after infection only in mice inoculated with live RSV. Compared with the infection caused by live RSV or attenuated UV light–treated RSV, mice inoculated with HI-RSV had no virus detected by culture, significantly decreased RSV RNA loads in the respiratory tract during the acute infection, and no RSV RNA detected during the chronic phase of the disease. In addition, these mice with no evidence of RSV RNA persistence had no objective signs of disease, as defined by lung HPS, Th-1/Th-2 cytokine expression, and pulmonary function test values, which were similar to findings in noninfected control animals.

Although we did not demonstrate the presence of RSV by culture during the chronic phase, RSV RNA was detected in 100% of mice inoculated with live RSV, which further emphasizes the need for active viral replication to establish RSV persistence. The correlation observed between RSV RNA persistence and chronic AHR likely reflects an association, rather than causation. Other mechanisms may also be involved. Sutton et al. [31] recently suggested that RSV persistence may be necessary but not sufficient for chronic inflammation and AHR. It can be argued that the infection and immune responses triggered initially by the virus contribute to the prolonged AHR and histopathologic changes observed in mice inoculated with live and attenuated RSV. It is also possible that the chronic changes observed in both AHR and histopathology are perpetuated by an immune response that has been reprogrammed and may be aberrant in its production of certain cytokines. Although findings of various studies in humans and animal models suggest that RSV infection elicits a type 2 immune response associated with the development of bronchiolitis and/or asthma [31–33], there is also evidence that IFN-γ responses, indicative of a Th-1 shift, play a significant role in RSV-induced AHR [25, 34, 35]. Indeed, we found that persistent infection with RSV was associated with increased lung IFN-γ mRNA expression and IFN-γ:IL-4 ratios, significant AHR, and chronic lung inflammation.

These findings are in agreement with previous observations in other experimental models. RSV can persist in epithelial cells for several passages [36] and can replicate in alveolar macrophages in vitro for at least 3 weeks after infection [37]. In immortalized macrophage cell lines, RSV produces persistent infection [10]. The persistence of genomic and messenger RSV RNA has been described in guinea pigs [14, 31, 38] and mice for several weeks after acute primary infection, despite the presence of specific humoral and cellular responses [11, 13, 39, 40]. Similarly, bovine RSV RNA was detected in calves for several weeks after the virus was no longer detected by culture [41]. In mice, Alvarez et al. found persistence of human metapneumovirus, an RNA virus closely related to RSV, despite the presence of neutralizing antibodies [42].

It appears unlikely for an RNA virus to persist without at least low-level replication in the host. This is supported by the findings of Schwarze et al., who were able to recover low-titer virus by culture in mice after treatment with anti-CD4 and anti-CD8 antibodies, 150 days after infection [11]. In guinea pigs, low-level replicating RSV recovered from whole lungs was associated with persistent lung inflammation on day 60 after inoculation [13]. Along with these findings we also showed that viral persistence was associated with chronic lung inflammation and increased IFN-γ expression, and, more importantly, a direct correlation between RSV RNA copy number and long-term AHR in mice inoculated with live RSV.

Human studies have suggested the possibility of RSV persistence. In adults with chronic obstructive pulmonary disease (COPD), RSV RNA was found at a greater frequency in the upper respiratory tract of patients with stable COPD than in those with acute exacerbations; indeed, RSV-positive patients with stable COPD had greater concentrations of plasma fibrinogen and IL-6 and faster deterioration of their pulmonary function at follow-up [15, 30, 43]. The persistence of RSV RNA in children has not been extensively studied. Postmortem specimens from children who died of sudden infant death syndrome showed by in situ hybridization that RSV was detected regardless of the season when the specimens were obtained, suggesting the possible persistence of RSV in the lungs of these infants [44].

In summary, the results of this study suggest that the chronic persistence of RSV and increased IFN-γ expression in the lungs likely contributed to the development of chronic airway disease, as demonstrated by persistent lung inflammation and AHR. These findings suggest a potential role for persistent infection in the pathogenesis of virus-induced asthma and open the possibility of new preventive and therapeutic interventions for those patients with virus-induced recurrent wheezing. The stage is ready for clinical studies to determine whether RSV persistence contributes to the establishment of chronic lung inflammation and the development of recurrent wheezing in children.

References


