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Recurrent Respiratory Syncytial Virus Infections in Allergen-Sensitized Mice Lead to Persistent Airway Inflammation and Hyperresponsiveness

Hiroto Matsuse,* Aruna K. Behera,* Mukesh Kumar,* Hamid Rabb, ‡ Richard F. Lockey,* and Shyam S. Mohapatra2*†

Respiratory syncytial virus (RSV) infection is considered a risk factor for bronchial asthma; however, the synergy between allergen sensitization and RSV infection in the development of pulmonary inflammation and asthma has been controversial. In this study the effects of primary and recurrent RSV infection on allergic asthma were examined in a group of control, RSV-infected, Dermatophagoides farinae (Df) allergen-sensitized, and Df allergen-sensitized plus RSV-infected BALB/c mice. Primary RSV infection in Df-sensitized mice transiently increases airway responsiveness, which is accompanied by increases in eosinophilic infiltration, the expression of ICAM-1, and macrophage inflammatory protein-1α (MIP-1α) in the lung tissue. A secondary RSV infection persistently enhances airway responsiveness in Df-sensitized mice, with a concomitant increase in MIP-1α and RSV Ag load in lung tissues. Bulk cultures of thoracic lymph node mononuclear cells demonstrate that acute RSV infection augments both Th1- and Th2-like cytokines, whereas secondary and tertiary infections shift the cytokine profile in favor of the Th2-like cytokine response in Df-sensitized mice. The elevated total serum IgE level in the Df-sensitized mice persists following only RSV reinfection. Thus, recurrent RSV infections in Df-sensitized mice augment the synthesis of Th2-like cytokines, total serum IgE Abs, and MIP-1α, which are responsible for persistent airway inflammation and hyperresponsiveness, both of which are characteristics of asthma. The Journal of Immunology, 2000, 164: 6583–6592.

Bronchial asthma is a chronic inflammatory disease of the airways characterized by pulmonary eosinophilia and airway hyperresponsiveness to various stimuli (1, 2). Exposure to primarily indoor allergens in infancy is a major risk factor for bronchial asthma (3, 4). Allergens induce a dominant Th2 response, characterized by the secretion of IL-4, IL-5, and IL-13 (5). The Th2-like cytokines play a critical role in the genesis of an allergic cascade, which may lead to persistent airway inflammation and asthma and involves the synthesis of several chemokines and adhesion molecules and the recruitment of eosinophils to the airway (5–7). The respiratory syncytial virus (RSV), commonly associated with lower lung infections in infancy, is known to exacerbate asthma (8–10). Viral infections stimulate cells to secrete a variety of cytokines that up-regulate airway inflammation and airway responsiveness (11). RSV infection has been reported to induce Th2-like cytokines and a specific IgE Ab response (12, 13). These results have led to the paradigm that allergen sensitization and RSV infection in infancy may synergistically augment the risk for the development of asthma. However, primary viral infections induce increased production of IFN-γ, which down-regulates a Th2-like response. Therefore, a RSV-induced Th2 response and the synergy between allergen sensitization and RSV infection have remained a paradox.

The mouse provides an excellent model to investigate the possibility of synergy between allergen sensitization and RSV infection at the immunologic level (14). The inhalation of dust mite allergens in the sensitized mouse induces eosinophil recruitment and the expression of cell adhesion molecules in lung tissue, leading to enhanced inflammation (15). Similarly, mice intranasally (i.n.) inoculated with human RSV develop lung disease characterized by pulmonary inflammation and airway responsiveness. Depending upon the dose of RSV and the age of the mice, they suffer either a mild infection with no overt signs of illness or a moderate/severe infection with weight loss, tachypnea, and patchy pneumonitis (16). The immunity to RSV infection in mice resembles the RSV-induced immunity found in humans (17, 18). RSV-infected mice exhibit increased production of IL-4 and eosinophils in lung tissues and an increase in airway hyperresponsiveness upon exposure to OVA (19), suggesting an interaction between RSV infection and allergen sensitization.

Genetically predisposed infants are exposed naturally to allergens in utero and may be exposed to allergens and RSV immediately following birth; we therefore hypothesized that RSV infection may act as a cofactor in allergy-predisposed infants and expedite the development of asthma. To test this hypothesis, we established a murine model of mite allergen sensitization and RSV

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3 Abbreviations used in this paper: RSV, respiratory syncytial virus; AR, airway responsiveness; AS, Df allergen-sensitized; ASRSV, Df allergen-sensitized plus RSV-infected; Df, Dermatophagoides farinae; HE, hematoxylin-eosin; i.n., intranasally; MCh, methacholine; MIP-1α, macrophage inflammatory protein-1α; MNC, mononuclear cell; P

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infection. In this model mice infected with only live RSV (5 × 10^5 PFU/mouse) exhibit RSV replication and lung pathology, but show no overt signs of illness (20). Analysis of cytokine mRNA expression in RSV-infected mice showed elevated expression of IL-6, IFN-γ, and eotaxin mRNAs in the lung; however, IL-10 and IL-13 mRNA expression increased in spleen cells, suggesting a bias toward Th2-like cytokine expression (21). Herein, we examined the effect of repeated RSV infection in allergen-sensitized mice and compared the airway reactivity, inflammation, and levels of ICAM-1, chemokines, cytokines, and IgE Abs in groups of control, RSV-infected, Dermatophagoides farinae (DF) allergen-sensitized, or DF allergen-sensitized and RSV-infected mice. The results indicate that repeated RSV infections play a critical role in the induction of persistently elevated pulmonary inflammation and a Th2-like response in allergen-sensitized mice.

Materials and Methods

**Mice and RSV infection**

Female BALB/c mice, 6–8 wk of age, and ICAM-1−/− and ICAM-1+/− C57BL6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in pathogen-free conditions at the James A. Haley Veterans Affairs Hospital Animal Center. The procedures were reviewed and approved by the University of South Florida and the James A. Haley Veterans Affairs Hospital committee on animal research. The A2 strain of human RSV (American Type Culture Collection, Manassas, VA) was propagated in HEp-2 cells (American Type Culture Collection) in a monolayer culture as previously described (22). Mice were infected by an i.n. inoculation of RSV suspension (5 × 10^5 PFU/50 μL/mouse) under light anesthesia as described previously (20, 21). All experiments were repeated at least twice.

**Immunization protocols**

Groups of mice were either RSV-infected or sham-infected with PBS or UV-inactivated RSV on days 4, 28, and 87 (Fig. 1). DF allergen-sensitized (AS) and DF allergen-sensitized plus RSV-infected (ASRSV) groups were immunized once i.p. on day −13 with 10 μg/mouse of DF allergens (Greer Laboratories, Lenoir, NC) in 1 mg/mouse aluminum hydroxide (alum). Then, these mice were challenged i.n., once daily for 3 consecutive days (days 1–3, 25–27, and 84–86), with 50 μg of DF in 50 μL of PBS/mouse. To facilitate the pulmonary aspiration of DF, mice were lightly anesthetized as described above for RSV inoculation. ASRSV mice were inoculated with RSV 24 h after the last i.n. challenge of DF on days 4, 28, and 87. Airway responsiveness (AR), serum total IgE, and DF-specific IgE and IgG1 Ab levels were determined in all groups of mice after 4, 10, and 17 days of primary and secondary RSV infections and 4 days following the tertiary infection. Lungs, spleens, and thoracic lymph nodes were removed from the mice 4 days after each RSV infection to determine RSV Ag loads, cytokines, and chemokines. Pathology and ICAM-1 immunohistochemistry in the lung tissues were only examined 4 days following the primary infection.

**Quantification of RSV Ag**

The total RSV Ag load in the lung homogenate was measured by an ELISA as previously described (20). Briefly, the supernatants of the lung homogenate were first incubated in goat anti-human RSV Ab (AB1128, Chemicon, Temecula, CA)-coated ELISA plates (Costar, Cambridge, MA) and then incubated with mouse anti-human RSV mAb (NCL-RSV3, Vector Laboratories, Burlingame, CA). Following incubation with peroxidase-labeled goat anti-mouse IgG Ab (Roche, Indianapolis, IN), immune complexes were detected using tetramethyl benzidine as the substrate. ODs were read at 450 nm using an automated ELISA reader. The results were considered positive if the mean OD of duplicate wells was >0.2 after subtraction of the background. The positive results were transformed to PFU per grams of lung by the standard curve obtained with virus of known titers (American Type Culture Collection).

**Determination of AR**

AR was measured in unoinfected mice by whole body plethysmography (Buxco, Troy, NY). As previously described (19), AR was expressed as an enhanced pause (P_{E20}), a calculated value that closely correlates with pulmonary resistance measured by a conventional, two-chamber plethysmograph in ventilated animals. Using a four-chamber plethysmograph and PBS- and OVA-sensitized mice, similar results were obtained in three independent experiments, suggesting the specificity and low variances of this method. Groups of mice (n = 4) were exposed for 5 min to nebulized PBS and subsequently to increasing concentrations (6–50 mg/ml) of nebulized methacholine (MCh; Sigma, St. Louis, MO) in PBS using the ultrasonic nebulizer. After each nebulization, recordings were taken for 5 min. The P_{E20} values measured during a 5-min sequence were averaged and are expressed for each MCh concentration as a percentage of baseline P_{E20} values obtained following PBS exposure.

**Immunohistochemical analysis**

Mice were sacrificed with an overdose (0.6 g/kg) of pentobarbital 4 days after the primary RSV inoculation or 5 days after the final DF challenge, and lung sections were subjected to paraffin embedding as previously described (20, 21). Lung inflammation was assessed after staining the section with hematoxylin and eosin (HE) and scoring for severity on a scale of 0–3 indicating the degree of inflammation. The entire lung section was reviewed, and pathological changes were evaluated for epithelial damage, peribronchovascular cell infiltrate, and interstitial-alveolar cell infiltrate for the mononuclear cells and polymorphs. Epithelial damage was scored as: 0 = no damage, 1 = increased cytoplasm of epithelial cells without desquamation, 2 = epithelial desquamation without bronchial exudate composed of inflammatory cells, and 3 = bronchial exudate composed of desquamated epithelial cells and inflammatory cells. Peribronchovascular cell infiltrate was scored as: 0 = no infiltrate, 1 = infiltrate up to four cells, 2 = infiltrate 5–10 cells, and 3 = infiltrate >10 cells. Interstitial-alveolar cell infiltrate was scored as: 0 = no infiltrate, 1 = mild, generalized increase in cell mass of the alveolar septa without thickening of the septa or significant airspace consolidation, 2 = dense septal infiltrate with thickening of septa, and 3 = significant alveolar consolidation in addition to interstitial inflammation. Pathological scores were expressed as the mean ± SEM. The numbers of eosinophils was determined in 10 peri-vascular areas/section under oil immersion lens. The slides were coded and scored in blind fashion twice each by different individuals. Intraobserver variation was <5%.

To examine ICAM-1 expression, slides were immunostained with ICAM-1 Ab. Briefly, paraffin-embedded sections were dewaxed in xylene and rehydrated in three graded concentrations of alcohol. Alcian blue was performed by incubating sections immersed in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven (twice, 5 min each time). Endogenous peroxidase was blocked with 0.3% H2O2 in methanol (30 min). The sections were then incubated with a hamster anti-mouse ICAM-1 mAb (clone 6584 EFFECT OF RECURRENT RSV INFECTIONS ON ASThma
RSV infection and RSV Ag load in BALB/c mice. a, Demonstration of RSV replication in the mouse lung 4 days after RSV infection. The expression of RSV mRNA was determined by RT-PCR, using primers for RSV N protein (22). Lanes 1 and 2, Sham-infected; lanes 3 and 4, UV-inactivated RSV. lanes 5 and 6, live RSV. RSV N mRNA is detected in lungs of live RSV inoculated mice. b, Immunohistochemical staining (dark) of RSV-infected perivascular cells in the mouse lung using polyclonal anti-RSV Ab. c, Total RSV Ag load in the lung tissue after repeated RSV infections. The mean (n = 6) ± SEM of total RSV Ag load in RSV and ASRSV mice 4 days after each RSV inoculation are shown. Neither control nor AS mice showed positive results. *p < 0.05 vs primary infection; †p < 0.05 vs RSV.

Analysis of cytokines and chemokines

Single-cell suspensions were prepared from the spleen (4 × 10^7/200 μl) and thoracic lymph node (2.5 × 10^7/200 μl) of BALB/c mice and cultured in wells coated with either an anti-CD3 Ab (1 mg/ml; clone 17A2, PharMingen) or Df allergen (100 μg/ml). The productions of IL-4, IL-5, and IFN-γ from 24-h (anti-CD3) or 48-h (Df) culture supernatant were determined by ELISA using matched Ab pairs (PharMingen) as previously described (23). To measure chemokine production, lungs were perfused with ice-cold PBS via the right ventricle, removed aseptically, and homogenized in 3 ml of lysis buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl_2, and 1 mM MgCl_2, pH 7.4). The homogenates were incubated on ice for 30 min, then centrifuged at 3000 rpm for 10 min. Supernatants were collected and passed through a 0.2-μm pore size filter (Gelman Sciences, Ann Arbor, MI). Concentrations of eotaxin, RANTES, and MIP-1α in the supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN). The results were expressed as the absolute chemokine amount per wet lung weight (picograms per milligrams of lung).

Quantification of total serum IgE and Df-specific serum Ig isotype

The total serum IgE level was determined by ELISA using a rat anti-mouse IgE Ab (clone R35-72, PharMingen) and biotin-conjugated rat anti-mouse IgE mAb (clone R35-92, PharMingen) as described previously (21). To measure the Df-specific serum Ig isotype, diluted sera were first incubated in Df extract (5 μg/ml)-coated ELISA plate followed by incubation with biotin-conjugated rat anti-mouse IgE mAb or IgG1 mAb (clone A85-1, PharMingen). The OD was read using an automatic ELISA plate reader at 405 nm. The total serum IgE level was expressed as micrograms per milliliter using mouse IgE standard (PharMingen). The Df-specific serum Ig levels were shown as an OD_{405} value.

Statistical analysis

Pairs of groups were compared by Wilcoxon rank-sum test. Differences between groups were considered significant at p < 0.05. Values for all measurements are expressed as the mean ± SEM. All analyses were performed on a Macintosh computer (Apple Computer, Cupertino, CA) with StatView II software (Abacus Concepts, Berkeley, CA).

Results

Repeated RSV exposure increases RSV Ag load in the lung

Intranasal administration of 50 μl of RSV (5 × 10^5 PFU/mouse) induces infection of lung cells that reaches a peak at 4–6 days after infection. N protein mRNA is detected in the lung tissues of all live RSV-injected BALB/c mice, but is not detected in sham- or UV-inactivated RSV-infected mice by RT-PCR on day 2 after infection (Fig. 2a). RSV Ag is detected immunohistochemically in lung tissues on day 4 only in RSV-infected mice using a polyclonal anti-RSV Ab (Fig. 2b) and not in sham- or UV-inactivated RSV infected mice (not shown). Total RSV Ag load in the lung tissues was determined by an ELISA 4 days after each RSV inoculation. No RSV Ag was detected in the control and AS groups of mice. Both RSV and ASRSV mice showed similar Ag loads after a primary infection. Following secondary infection, the level of RSV Ag load in the lung homogenate of both RSV and ASRSV mice significantly increased compared with that after the primary infection (Fig. 2c). However, after the tertiary infection, only RSV-infected mice showed a decline in the level of RSV Ag load in their lung tissue, whereas ASRSV mice showed a significant increase in the RSV Ag load. Plaque assays were also performed in mice 4 days after infection; the number of plaques in RSV and ASRSV mice were similar (mean, 2 × 10^3 to 10^4 PFU/g lung; data not shown).
RSV infection enhances airway responsiveness in Df-sensitized mice

The airways of RSV and AS mice, 4 days after primary RSV infection and 5 days after the final Df challenge, were significantly more reactive to 50 mg/ml of MCh than were control mice. ASRSV mice showed a significant increase in MCh responsiveness compared with all other groups (Fig. 3a). The increase in AR in RSV mice was observed even 10 days after primary infection and returned to a normal level by day 17. In addition, the secondary RSV infection increased AR 4 days after inoculation, but returned to a normal level by day 10. The tertiary RSV infection slightly, but not significantly, increased AR 4 days after the inoculation (Fig. 3b). ASRSV, compared with AS mice, also exhibited a significant increase in AR 4 days after the secondary infection. In contrast to the primary RSV infection, this increase in AR in the ASRSV mice was detectable on day 17 after the secondary inoculation (Fig. 3c). These results suggest that recurrent RSV infections attenuate AR in control mice, whereas they increase AR in allergen-sensitized mice.

Allergen sensitization and RSV infection together enhance lung inflammation

Lung inflammation was examined 4 days after the primary RSV infection or 5 days after the final Df challenge by staining with HE or anti-mouse ICAM-1 Abs. Representative pathological features of HE staining are shown in Fig. 4. Epithelial shedding, mucous plugging, and mononuclear cell (MNC) infiltration were observed in RSV mice (Fig. 4B) but not in controls (Fig. 4A). Lungs of AS mice exhibited goblet cell metaplasia and cellular infiltration with MNC and eosinophils (Fig. 4C). A bronchial exudate, composed of both MNC and eosinophils, and massive tissue eosinophilic infiltration were found in ASRSV mice (Fig. 4D). A semiquantitative analysis using a scoring system for inflammatory cells in the lung is shown in Table I. The mean number of infiltrating eosinophils per 10 perivascular areas in ASRSV mice was significantly higher than that in AS mice (AS, 353.6 ± 16.1 cells; ASRSV, 769.0 ± 20.0 cells; p < 0.01). The results indicate that the lungs of ASRSV mice show evidence of additive and synergistic effects for epithelial damage and cellular infiltration, respectively.

ICAM-1 plays a role in allergen sensitization and RSV infection-induced lung inflammation

Fig. 5A shows the results of an immunohistochemical analysis for ICAM-1 in mouse lung tissues. The number of ICAM-1-positive cells per 200 epithelial cells increased significantly in both RSV

FIGURE 3. RSV infection enhances AR to MCh in Df allergen-sensitized mice. a, AR to inhaled MCh (6–50 mg/ml) was measured 4 days after the primary RSV inoculation or 5 days after the final Df challenge in the four groups of BALB/c mice. Results are expressed as the mean (n = 8) ± SEM of P<sub>enh</sub> values. * p < 0.05 vs control; †, p < 0.01 vs all other groups. b and c, Time course of AR after primary and repeated RSV infections. AR was measured 4, 10, and 17 days after the primary RSV inoculation, similarly after the secondary inoculation, and only 4 days following the tertiary inoculation. Results are expressed as the mean (n = 8) ± SEM of P<sub>enh</sub> values at 50 mg/ml of MCh in control and RSV (b) and AS and ASRSV (c) mice. * p < 0.05; †, p < 0.01.

FIGURE 4. RSV- and Df allergen-induced lung inflammation. Lung tissue was removed from BALB/c mice 4 days after the primary RSV inoculation or 5 days after the final Df challenge and was stained with HE. Representative photomicrographs from each group (n = 6 for each) are shown. A, Control; B, RSV; C, AS; D, ASRSV mice. a, airway; v, vessel. Arrows indicate cellular infiltration.
and AS mice compared with that in control mice (control, 5.0 ± 1.8 cells; RSV, 30.0 ± 4.3 cells; AS, 40.8 ± 2.0 cells; p < 0.01 both), ASRSV (80.0 ± 20 cells), compared with AS, mice exhibited a 2-fold increase in ICAM-1-positive epithelial cells. Fig. 5B shows the quantitation of inflammation in the lungs of both ICAM-11+/+ and ICAM-1−/− mice. The ICAM-1−/− mice exhibited significantly lower inflammation in all counts than the ICAM-11+/+ mice, suggesting an important role of ICAM-1 in inflammation.

**Table I. Change in lung pathology following mite-sensitization and/or RSV infection**

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Control</th>
<th>RSV</th>
<th>Df</th>
<th>Df + RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelium Ep. Shedding</td>
<td>1.7 ± 0.2</td>
<td>18.3 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.7 ± 1.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>19.7 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC metaplasia</td>
<td>0.8 ± 0.2</td>
<td>2.2 ± 0.5</td>
<td>23.3 ± 1.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>21.7 ± 1.6&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interstitial-alveolar infiltrate</td>
<td>MNC</td>
<td>3.3 ± 0.3</td>
<td>16.7 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.7 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PMNC</td>
<td>1.7 ± 0.2</td>
<td>4.8 ± 1.2</td>
<td>8.3 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peribronchovascular infiltrate</td>
<td>MNC</td>
<td>1.7 ± 0.2</td>
<td>17.3 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.3 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PMNC</td>
<td>0.8 ± 0.1</td>
<td>5.0 ± 1.2</td>
<td>16.7 ± 1.9&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean (n = 6) ± SEM (p < 0.05) compared with control (<sup>b</sup>), RSV-infected mice (<sup>c</sup>), or mite-sensitized mice (<sup>d</sup>).

**FIGURE 5.** A, Immunohistochemistry for mouse ICAM-1 in lung tissue. Paraffin-embedded lung tissue obtained from BALB/c mice 4 days after the primary RSV inoculation or 5 days after the final Df challenge was stained with anti-mouse ICAM-1 Ab in control (A), RSV (B), AS (C), and ASRSV (D) mice. Representative photographs from each group (n = 6 for each) are shown. a, airway; v, vessel. Arrows indicate positive staining for ICAM-1 on airway epithelial cells. B, Semiquantitative analysis of pathological changes in ICAM-11+/+ and ICAM-1−/− mice after primary RSV infection. The bars represent the mean ± SEM; the asterisk indicates the level of significance at p < 0.05. ○, sham-infected ICAM-11+/+; ●, sham-infected ICAM-1−/−; △, RSV-infected ICAM-11+/+; ▲, RSV-infected ICAM-1−/−.
**EFFECT OF RECURRENT RSV INFECTIONS ON ASTHMA**

Recurrent RSV infections enhance the Th2-like cytokine response in Df allergen-sensitized mice

Table II shows cytokine production from anti-CD3-stimulated thoracic lymph node cultures. The primary RSV infection produced maximal levels of IL-4, IL-5, and IFN-γ, with their amounts decreasing following secondary and tertiary infections. In marked contrast, all Df challenges increased IL-4 and IL-5, but not IFN-γ. Compared with AS mice, ASRSV mice exhibited significant reductions in IL-4 and IL-5 after primary RSV infection. Secondary and tertiary RSV infections increased IL-4 and IL-5 in ASRSV mice. Primary infection in AS mice decreased IFN-γ, but increased IL-4 and IL-5, whereas secondary and tertiary infections induced relatively less IFN-γ and higher levels of IL-4 and IL-5, leading to a Th2 bias. The ratio of IL-4 to IFN-γ and that of IL-5 to IFN-γ produced in anti-CD3-stimulated thoracic lymph node cultures from four groups of mice is shown in Fig. 7. Primary RSV infection induced significant increases in the IL-4:IFN-γ and IL-5:IFN-γ ratios and drastic reductions in these ratios after secondary and tertiary infections (Fig. 7, a and b).

Table III shows the Df-allergen specific cytokine production from thoracic lymph node cell cultures. IL-4 and IL-5 were detected in AS and ASRSV mice, but not in control and RSV mice. Compared with AS mice, ASRSV mice exhibited significant increases in Df allergen-specific IL-4 and IL-5 productions only after

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**Table II. Cytokine production from anti-CD3-stimulated thoracic lymph node MNC**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RSV (1)</th>
<th>AS (2)</th>
<th>ASRSV (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-4 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>17.8 ± 2.2</td>
<td>131.6 ± 234.5</td>
<td>567.6 ± 65.3</td>
<td>154.4 ± 16.9</td>
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<tr>
<td>Secondary</td>
<td>11.4 ± 1.9</td>
<td>27.6 ± 6.9</td>
<td>43.9 ± 5.9*</td>
<td>345.3 ± 25.6*</td>
</tr>
<tr>
<td>Tertiary</td>
<td>10.2 ± 1.5</td>
<td>105.4 ± 9.8*</td>
<td>221.0 ± 16.5</td>
<td>2007.0 ± 210.3</td>
</tr>
<tr>
<td><strong>IL-5 (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>5.2 ± 0.4</td>
<td>340.8 ± 39.7</td>
<td>305.2 ± 45.3</td>
<td>120.6 ± 19.6</td>
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<tr>
<td>Secondary</td>
<td>4.9 ± 0.3</td>
<td>13.3 ± 5.0</td>
<td>132.6 ± 9.9</td>
<td>1362 ± 152.3</td>
</tr>
<tr>
<td>Tertiary</td>
<td>4.2 ± 0.3</td>
<td>27.2 ± 3.6*</td>
<td>1050.8 ± 156.3</td>
<td>5911.2 ± 465.2</td>
</tr>
<tr>
<td><strong>IFN-γ (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>144.6 ± 9.8</td>
<td>2568.4 ± 269.8</td>
<td>108.2 ± 9.3</td>
<td>423.4 ± 25.6*</td>
</tr>
<tr>
<td>Secondary</td>
<td>199.1 ± 15.6</td>
<td>847.2 ± 98.8</td>
<td>242 ± 27.9</td>
<td>601.5 ± 55.2</td>
</tr>
<tr>
<td>Tertiary</td>
<td>88.9 ± 6.7</td>
<td>1524.6 ± 145.9</td>
<td>142.8 ± 11.8</td>
<td>1398.8 ± 205.9</td>
</tr>
</tbody>
</table>

*Thoracic lymph node MNC obtained from control, RSV-infected (RSV), AS, and ASRSV mice 4 days after each RSV infection were cultured in the presence of anti-CD3 molecular complex for 24 h. The concentrations of IL-4, IL-5, and IFN-γ in culture supernatants were determined by ELISA. Results are shown as mean (n = 8 for each group) ± SEM.

*a, p < 0.05 vs control; †, p < 0.01 vs control.
repeated RSV infections, IFN-γ was detected in all groups at comparable levels. After primary and tertiary RSV infections, ASRSV mice showed a significant increase in Df allergen-stimulated IFN-γ production compared with AS mice. In contrast, primary RSV infection in AS mice did not alter the IL-4:IFN-γ and IL-5:IFN-γ ratios; however, secondary and tertiary infections in these mice resulted in significantly higher IL-4:IFN-γ and IL-5:IFN-γ ratios in ASRSV mice compared with AS mice (Fig. 7, c and d). Together, these results suggest a shift toward a Th2-like response after repeated exposure to RSV.

**Discussion**

A key finding of this study is that recurrent RSV infection plays an important role in augmenting airway hyperresponsiveness, the hallmark of asthma, in a murine model of allergy. Our results indicate a number of potential mechanisms underlying the RSV-induced enhancement of asthma in this model. RSV infection increases the expression of ICAM-1 and the number of eosinophils recruited into the lung; recurrent RSV infection, especially, induces an elevated expression of MIP-1α in the airway. Furthermore, recurrent RSV infections shifted T cell responses from a Th0-like response after primary infection toward a Th2-like response after tertiary infection. This was accompanied by increases in the RSV Ag load in the lung and the IgE levels in the serum. Together, these RSV infection-induced changes in the lungs of an allergen-sensitized mouse may contribute to the development of chronic asthma.

Our results demonstrate that the primary RSV infection in normal mice increases AR; however this increase is transient, as recurrent infections do not lead to any further enhanced AR. In marked contrast, in AS mice, after primary infection AR remained

### Table III. Cytokine production from Df-stimulated thoracic lymph node MNC

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RSV (1)</th>
<th>AS (2)</th>
<th>ASRSV (3)</th>
<th>Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>13.7 ± 2.0*</td>
<td>41.2 ± 8.9*</td>
<td>&lt;0.01 NS</td>
</tr>
<tr>
<td>Secondary</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>6.2 ± 1.1*</td>
<td>31.7 ± 5.6†</td>
<td>&lt;0.01 &lt;0.05</td>
</tr>
<tr>
<td>Tertiary</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>72.2 ± 6.3†</td>
<td>302.4 ± 26.4°</td>
<td>&lt;0.01 &lt;0.05</td>
</tr>
<tr>
<td>IL-5 (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>6.3 ± 1.4†</td>
<td>12.0 ± 9.9†</td>
<td>&lt;0.01 NS</td>
</tr>
<tr>
<td>Secondary</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>5.6 ± 0.3*</td>
<td>468.1 ± 32.6°</td>
<td>&lt;0.01 &lt;0.01</td>
</tr>
<tr>
<td>Tertiary</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>703.5 ± 66.9°</td>
<td>2205.9 ± 198.7°</td>
<td>&lt;0.01 &lt;0.05</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>240.2 ± 26.3</td>
<td>261.1 ± 32.6</td>
<td>367.2 ± 9.8</td>
<td>743.4 ± 82.5°</td>
<td>&lt;0.05 &lt;0.05</td>
</tr>
<tr>
<td>Secondary</td>
<td>161.3 ± 19.6</td>
<td>204.6 ± 25.3</td>
<td>185.7 ± 15.6</td>
<td>261.3 ± 22.6</td>
<td>NS NS</td>
</tr>
<tr>
<td>Tertiary</td>
<td>138.6 ± 16.7</td>
<td>160.3 ± 11.9</td>
<td>19.4 ± 6.7</td>
<td>488.0 ± 59.6*</td>
<td>NS &lt;0.01</td>
</tr>
</tbody>
</table>

*Thoracic lymph node mononuclear cells obtained from control, RSV, AS, and ASRSV mice 4 days after each RSV infection were cultured in the presence of Df allergen for 48 h. The concentrations of IL-4, IL-5, and IFN-γ in culture supernatants were determined by ELISA. Results are shown as mean (n = 8 for each group) ± SEM.

* p < 0.05 vs control; †, p < 0.01 vs control; ‡, p < 0.05.
unchanged, while significant increases in AR were noted after secondary and tertiary infections. The differences compared with a previous report (21) were primarily noted with higher concentrations of Mch. This may be due to differences in the sensitization protocol, the allergen, the dose of virus, or the interval between the final challenge and measurement period. Our results are consistent with those of the epidemiological studies, which demonstrated that recurrent RSV infection is common in infancy (24). Also, two or more episodes of wheezing and lower respiratory tract illness are related to an increased risk of subsequent abnormal lung function (25).

The expression of ICAM-1 may constitute one of the most important mechanisms underlying the synergy between RSV infection and allergen sensitization. Df-sensitized and RSV-infected mice, compared with only Df-sensitized mice, showed a significant increase in ICAM-1-positive epithelial cells after the primary RSV infection. Consistent with previous reports (26, 27), both RSV infection and Df sensitization induced the expression of ICAM-1 in epithelial cells. ICAM-1 is implicated in the induction of Ag-induced, airway inflammation and AR in primate and murine models (7, 28). ICAM-1 knockout mice do not show increased AR following RSV infection (29), suggesting an important role of ICAM-1 in RSV-induced AR. Epithelial cells also enhance ICAM-1 expression after exposure to allergens or to RSV via the induction of inflammatory cytokines (26). We have recently found that ICAM-1 facilitates the initiation of RSV infection of epithelial cells (A. K. Behera, H. Matsuse, M. Kumar, X. Kong, F. Lockey, and S. S. Mohapatra, unpublished data). Together, these results indicate that ICAM-1 may constitute a pivotal player in furthering pulmonary inflammation during concomitant allergen sensitization and RSV infection.

Chemokines, such as MIP-1α, RANTES, and eotaxin, play important roles in a variety of immune and inflammatory responses, specifically, eosinophil migration (6). Our results demonstrate that primary RSV infection enhances the production of RANTES and MIP-1α, but not eotaxin, in lung tissue. Primary RSV infection in AS mice enhances MIP-1α. These results are consistent with human studies in which RSV enhanced RANTES and MIP-1α production in cultured epithelial cells in vitro (30). One human study reported a significant increase in MIP-1α in nasopharyngeal secretions following RSV infection (R. C. Welliver, American Academy of Allergy and Immunology Meeting, 1999). A noteworthy distinction between Df and RSV exposure is that Df challenges induced eotaxin, whereas recurrent RSV infection induced enhanced RANTES production. Interestingly, a significant increase in MIP-1α was found in ASRSV mice compared with AS or RSV-infected mice, which suggests the existence of a potential mechanism of synergy between allergen sensitization and RSV infection. The mechanism of the increased production of these chemokines upon exposure to RSV and/or AS may involve a transcriptional up-regulation or might simply reflect a greater number of cells in the airway rather than an increase in chemokine production on a per cell basis. Because the number and type of cells in the lung tissue were not determined in this study, the basis for MIP-1α remains unclear.

Another important facet of this study is the immune response to RSV vis-à-vis allergen exposure. Our results clearly demonstrate that the primary infection, in the absence of allergen sensitization, induces an aggressive cytokine response, typical of a Th0-like response, and that further infections induce decreased Th2 or increased Th1 dominance. However, in allergen-sensitized mice, the primary infection induced IFN-γ production, which down-regulated Th2-like cytokines. Further infections of these mice reversed the cytokine dominance from Th1-like to Th2-like. Similarly, a comparison between RSV and ASRSV mice demonstrated

FIGURE 8. Time course of total serum IgE and Df-specific serum Ig isotype after primary and repeated RSV infections. The mean (n = 8) ± SEM of total serum IgE (a) and Df-specific Ig isotype (IgE (b) and IgG1 (c)) in four groups of BALB/c mice 4, 10, and 17 days after the primary RSV inoculation, similarly after the secondary inoculation, and only 4 days following the tertiary RSV inoculation are expressed. *, p < 0.05; †, p < 0.01 (vs AS).
that a primary allergen challenge in RSV mice suppresses both Th1- and Th2-like responses, while repetitive allergen challenges induce an increase in the generalized Th2-like response, but not in the Th1-like response, in ASRSV mice.

Consistent with the Th2-like response, primary RSV infection enhances total serum IgE levels in AS mice, and the magnitude of increase is statistically significant. The increase in total serum IgE is higher after the secondary infection compared with that detected after the primary infection. Thus, the pattern of total serum IgE is different between AS and ASRSV mice. This suggests that the enhanced IgE response observed in ASRSV mice is probably due to the increase in IgE Abs specific to RSV. This inference is consistent with previous reports that RSV is capable of inducing an IgE class switch (31, 32). The RSV G protein especially mounts an IgE response (33, 34).

Several investigators reported allergen-specific IgE enhancement in animals with prior RSV (35, 36), influenza (37), or parainfluenza-3 virus (38) infection, suggesting that the virus-induced increase in allergen-specific IgE is possibly due to the facilitation of allergen uptake by virally damaged epithelial cells. It is likely that an increase in IgE-specific IgE was not detected in the present study because mice were first sensitized and challenged with allergen and subsequently infected with RSV.

In conclusion, the effect of RSV infection varies depending upon the inflammatory context of the lung. Both primary and recurrent RSV infections contribute to and augment ongoing allergic inflammation; however, in the absence of allergic sensitization, the effects of RSV are transient. The finding that levels of RSV Ag, MIP-1α, and Th2-like cytokines increase upon recurrent infections is in agreement with the report that MIP-1α increases the preponderance of Th2-like cytokines (39). MIP-1α not only promotes eosinophilia and pulmonary inflammation, but also delays RSV clearance from lung tissue (40). Our results show that allergen sensitization and/or RSV infection up-regulate the expression of several molecules, either additively or synergistically, which leads to the persistence of inflammation and, consequently, airway hyperreactivity. Thus, RSV infection increases ICAM-1 expression on nasal epithelial cells (41) and MIP-1α in the lung tissue, which may enable allergic inflammation by augmenting lymphocyte recruitment. Primary infection also induces IFN-γ, which has been reported to increase the survival of eosinophils and thus enhance inflammation in the lung (42). Recurrent RSV infections produce less IFN-γ and enhance a generalized Th2-like response, specifically with increased IL-5 production. Th2 cells are involved in the pathogenesis of asthma, resulting in eosinophilia and mucous hypersecretion and airway hyperresponsiveness (43). Overall, recurrent RSV infections play a critical role in the development of persistent inflammation and airway hyperresponsiveness in individuals with genetic predisposition to atopy and, therefore, constitute a major risk factor for asthma.

References


