Immune Interaction between Respiratory Syncytial Virus Infection and Allergen Sensitization Critically Depends on Timing of Challenges

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Severe respiratory syncytial virus (RSV) infection has been hypothesized to be a risk factor for the development of allergy and asthma, but epidemiologic studies in humans have been inconclusive. By use of a well-characterized murine model of RSV infection and allergic sensitization with ovalbumin, the effect of a preceding severe RSV infection on the development of the pulmonary allergic inflammatory response and airway hyperresponsiveness (AHR) was tested. The impact of prior allergic sensitization on RSV-induced illness, as measured by weight loss, was also evaluated. RSV infection before allergic sensitization decreased allergen-induced AHR, production of interleukin-13 in lung tissue, and lung eosinophilia. In contrast, allergic sensitization before RSV infection increased AHR and decreased RSV-related weight loss and lung levels of interferon-γ but did not alter viral clearance. These data provide evidence that RSV-associated AHR occurs in hosts with allergic responses and that allergic inflammation is diminished when preceded by RSV infection.

In some studies, respiratory syncytial virus (RSV) infection is highly associated with the development of asthma and allergic sensitization in children [1, 2]. Severe RSV bronchiolitis during infancy was found to increase the prevalence of both asthma and allergic sensitization in children up to age 7.5 years [2]. Other studies show no association between RSV infection and an increased risk for developing allergic disease [3, 4]. Therefore, it is unclear whether severe RSV infection predisposes to the development of allergy and airway disease or whether allergic disease predisposes to more severe respiratory symptoms from RSV infection.

Several recent studies suggest that infection with some pathogens early in life may protect against the subsequent development of allergic symptoms [5–7]. A possible protective effect of early viral infection may be the strong interferon (IFN) response induced by most viral pathogens [8]. IFN-γ, in particular, may skew the CD4 T lymphocyte profile toward a type 1 profile and inhibit the development of type 2 responses to allergens [8]. By reducing the capacity to mount a type 2 response, viral infections may decrease the development of allergic sensitivity and asthma [8]. Our work and that of others show that primary RSV infection in BALB/c mice induces a dominant type 1 cytokine profile [9–11]. On the basis of these findings, we hypothesized that the allergic phenotype in mice with RSV infection before allergic sensitization with ovalbumin (OVA) would be reduced in comparison with that of noninfected mice that had been allergically sensitized. Viral infections are exacerbating factors for preexisting allergic asthma [12]. Therefore, we also hypothesized that mice with RSV infection after allergen sensitization would have airway hyperresponsiveness (AHR), compared with allergically sensitized mock-infected mice. Both of our hypotheses were tested in a well-characterized murine model of allergic sensitization with OVA by varying the time course of intranasal (inl) infection with RSV [10, 13, 14].

Methods

Study protocol. Two protocols were used, with 2 groups of mice in each protocol (figure 1). The pathogen-free 8-week-old female BALB/c mice were purchased from Charles River Laboratories. In the RSV before OVA protocol, the RSV-OVA group was infected inl with 106 plaque-forming units of RSV on day 0, and the mock-OVA group was mock infected inl with culture media on the same day. Lungs from 4 mice in each group were excised to obtain plaque-forming units on day 4, the day of peak viral replication [15]. On day 14, both groups were injected intraperitoneally (ip) with 0.1 mL (10 mg) of chicken OVA, grade V (Sigma Chemical), complexed with 2 mg of Al(OH)3, as described elsewhere [13]. For 8 days (days 28–35), the mice were placed in an acrylic box and were exposed to aerosols of 1% OVA diluted in sterile PBS dispersed by an ultrasonic nebulizer (Ultraneb 99; DeVilbiss) for 40 min each day. On day 37, mice were killed, and specimens were obtained from mice for histopathology (4 mice), cytokine measure-
RSV before OVA protocol

| RSV or | OVA | Methacholine challenge, cytokines, histopath
|-------|-----|----------------------------------------
| Day 0  | ip  |                                         |
| Day 4  | pfu |                                         |
| Day 14 |     |                                         |
| Day 28 |     |                                         |
| Day 35 |     |                                         |
| Day 36 |     |                                         |

OVA before RSV protocol

| OVA | RSV or Mock | Methacholine challenge, Histopath
|-----|-------------|----------------------------------------
| Day 0 | ip |                                 |
| Day 14 |   |                                 |
| Day 21 |   |                                 |
| Day 39 |   |                                 |
| Day 49 |   |                                 |

Figure 1. Time line of experimental protocol. Histopath, histopathologic analysis; Mock, mock infection; OVA, ip, intraperitoneal injection of ovalbumin formulated with Al(OH)3; pfu, viral replication in plaque-forming units; RSV, respiratory syncytial virus infection.

In the OVA before RSV protocol, on day 0, the OVA-RSV and OVA-mock groups were injected ip with 0.1 mL (10 mg) of chicken ovalbumin, grade V, complexed with 2 mg of Al(OH)3, as described above. On days 14–21, mice were placed in an acrylic box and were exposed to aerosols of 1% OVA diluted in sterile PBS dispersed by an ultrasonic nebulizer (Ultraneb 99) for 40 min each day. The OVA-RSV group was infected with 10^7 pfu of RSV on day 35; the OVA-mock group was mock infected with culture medium on the same day. Lungs from 4 mice in each group were excised for plaque-forming units and cytokines on days 39 and 41, the days of peak viral replication and peak IFN-γ response, respectively. On day 49, mice were killed for histopathology (4 mice) and methacholine challenge and BAL cell counts and differentials (8 mice).

Cells and virus. RSV strain A2 was provided by R. Chanock (NIH). Master stocks and working stocks of RSV were prepared as described elsewhere [15].

Mouse infection. On day 0 in the RSV before OVA protocol and on day 35 in the OVA before RSV protocol, mice were infected with RSV or were given mock-infected culture medium inl, as described elsewhere [15].

Methacholine challenge. Mice were anesthetized with ip injections of pentobarbital sodium (85 mg/kg), and a tracheostomy tube was placed. The internal jugular vein was cannulated, and a microsyringe was attached to intravenous tubing for methacholine administration. The mice then were placed in a whole body plethysmography chamber and were mechanically ventilated. Changes in lung resistance and compliance were measured as described elsewhere [13].

Protocol for examining lung sections. The lung sections were prepared and examined by one observer in a blinded fashion, as described elsewhere [14].

Quantitation of interleukin (IL)–5, IL-13, and IFN-γ in lung tissues. Levels of IL-5, IL-13, and IFN-γ in lung tissues of the 4 groups of mice were measured by commercial ELISA kits (R&D Systems), according to the manufacturer’s protocols. Using ground lung supernatants, lungs from each group were analyzed for cytokine levels, as described elsewhere [14].

Statistical analysis. Results are expressed as mean ± SEM. Results of histopathologic analysis of cellular composition of the lung, measurements of cytokines, weight loss, and dose-response curves to methacholine were compared by analysis of variance. Differences were considered to be significant at P < .05.

Results

Prior RSV infection decreases allergen-induced AHR. To determine the effect of prior RSV infection on the development of allergen-induced AHR, methacholine challenges were performed on the RSV-OVA and mock-OVA groups on day 36, as outlined in the RSV before OVA protocol (figure 1). At the final dose of methacholine, 3700 μg/kg, the mock-OVA group had significantly greater lung resistance than the RSV-OVA group (16.2 ± 2.3 vs. 5.7 ± 0.9 cm of H2O/ml/s, respectively; P < .05; figure 2). Thus, RSV infection before allergen sensitization inhibited the development of allergen-induced AHR.

Allergen sensitization before RSV infection increases RSV-induced AHR. To determine the effect of RSV infection on AHR after allergen sensitization, methacholine challenges were performed on the RSV-OVA and OVA-mock groups on day 49 in the OVA before RSV protocol (figure 1). The OVA-RSV group had significantly greater AHR than the OVA-mock group (5.3 ± 1.8 vs. 2.8 ± 0.5 cm of H2O/ml/s, respectively; P < .05; figure 3). Therefore, RSV infection after allergic sensitization increases AHR.

Allergen sensitization before RSV infection reduces RSV-induced weight loss. In addition to AHR, weight loss is another measure of RSV-induced pathophysiology. Therefore, we weighed mice in the OVA-RSV and RSV groups daily after RSV infection (figure 4). The OVA-RSV group had significantly
RSV infection after allergen sensitization results in increased numbers of lung lymphocytes. Histologic analysis was done on day 49 in the OVA before RSV protocol, to examine the composition of lung inflammation as a result of RSV infection after allergen sensitization (figure 5B). The OVA-mock and OVA-RSV groups had lung lymphocytosis (2+) in the bronchovascular compartments; only the OVA-RSV group had lung lymphocytosis (2+) in the perivenous compartment. No eosinophils were seen in either group at this time point.

Prior RSV infection decreases allergen-induced lung IFN-γ. We next measured the concentration of IFN-γ in lung supernatants of the mock-OVA group was 133.8 ± 32.0 pg/mL, compared with <62.5 pg/mL (the limit of detection in this assay) in all samples from the RSV-OVA group. This difference in amounts of IFN-γ in the lung supernatants between the 2 groups was statistically significant (P < .05). Therefore, RSV infection before allergen sensitization decreases a type 2 cytokine that affects the physiologic response to allergic inflammation in the lung. IL-5 levels were measured in lung supernatants of both groups but were undetectable.

Allergen sensitization before RSV infection decreases level of RSV-induced IFN-γ. We next measured the concentration of IFN-γ in lung supernatants of the mock-OVA group was 133.8 ± 32.0 pg/mL, compared with <62.5 pg/mL (the limit of detection in this assay) in all samples from the RSV-OVA group. This difference in amounts of IFN-γ in the lung supernatants between the 2 groups was statistically significant (P < .05). Therefore, RSV infection before allergen sensitization decreases a type 2 cytokine that affects the physiologic response to allergic inflammation in the lung. IL-5 levels were measured in lung supernatants of both groups but were undetectable.

Prior RSV infection decreases allergen-induced lung eosinophilia. Histology was done on day 35 in the OVA before RSV protocol, compared with the OVA-RSV group (2178 ± 227.7 vs. 278.5 ± 142.6 pg/mL, respectively; P = .0004). Allergen sensitization before RSV infection
Figure 5. A. Tabulation of histologic analysis showing numbers of eosinophils (eos) and lymphocytes (lymphs) and their distributions in bronchovascular (BV) and perivenous (PV) locations. A, respiratory syncytial virus (RSV)-ovalbumin (OVA) and mock-OVA groups on day 36 of protocol “RSV before OVA.” *P < .05 vs. RSV-OVA group. B, OVA-RSV and OVA-mock groups on day 49 of protocol. *P < .05 vs. OVA-mock group.

Discussion

Epidemiologic studies investigating the effect of RSV infection on the development of allergic inflammation and asthma have produced conflicting results [1–4]. RSV infection in young children that is so severe as to require hospitalization for respiratory failure has been cited as a predisposing factor for the development of allergic disease [2]. An alternative possibility is that RSV bronchiolitis is more severe in children with the allergic phenotype. We used a well-characterized murine model [10, 13, 14] to determine the effect of RSV on the development of the allergic phenotype and to examine the effect of prior allergic sensitization on subsequent RSV infection. We found that RSV infection 2 weeks before allergic sensitization significantly decreased allergen-induced AHR, IL-13 production, and eosinophilic inflammation in the lung, when compared with mock-infected, allergically sensitized mice. In contrast, RSV infection after allergic sensitization led to significantly increased AHR. This is of particular interest, since we showed earlier that RSV infection alone has no effect on AHR [13]. The interaction between viral infections and allergic disease is currently an area of intense investigation and controversy. The "hygiene hypothesis," an explanation for the increase in allergic disease over the past 30 years, postulates that infections in very early life that result in an intense type 1 immune response are protective against the subsequent development of atopy [17–19]. Japanese schoolchildren with a positive tuberculin response had a lower rate of asthma development and lower serum IgE levels and blood cytokine levels biased toward a type 1 profile than children who had a diminished delayed hypersensitivity response to Mycobacterium tuberculosis [5]. Infection with measles virus [20] and hepatitis A virus [21] seem to be protective against the development of allergic disease in epidemiologic studies. Viral and bacterial infections during infancy also may prevent the development of allergic disease. For instance, children at increased risk of infection, specifically, young children with ≥1 older sibling and children who attend day care within the first 6 months of life, are protected against the development of asthma [6].

To our knowledge, our results are the first to show in a murine model that RSV infection before allergic sensitization with OVA protects against the development of the allergic phenotype. Other animal studies also conclusively show that viral infections protect against the development of type 2 inflammation characteristic of allergic disease. Specifically, prior influenza virus lung infection protects against the eosinophilic lung inflammation and type 2 cytokine secretion that occurs when RSV attachment protein immunization is followed by RSV infection [22]. This protective effect was long lived when spleen cells from influenza-infected mice were transferred to influenza-naive mice [22]. Of particular relevance to our studies, prior RSV infection prevented the enhanced pulmonary inflammatory response seen after RSV challenge in BALB/c mice immunized with formalin-inactivated RSV [23]. Both the RSV attachment protein and formalin-inactivated RSV vaccine models, similar to allergic disease, are dependent on production of type 2 cytokines.

Our results are inconsistent with those of Schwarze et al. [24], who reported that RSV infection in BALB/c mice before allergic sensitization causes increased allergic inflammation. This group reported that primary RSV infection in BALB/c mice, independent of allergen sensitization, causes AHR and induces the production of IL-5 and eosinophilia [24]. However, we and others have found that BALB/c mice do not produce significant eosinophilia and instead have an IFN-γ response after primary RSV infection [10, 25–29]. The methodologic differences between our findings and those of Schwarze et al. are that we used a virus inoculum of 10^7 pfu and documented RSV replication in the lung; Schwarze et al. used a 10^6 pfu inoculum and did not document replication of RSV in lung tissue [24].

Although we determined that RSV infection before allergic sensitization decreased AHR, we found increased AHR when RSV infection occurred after allergic sensitization. We reported earlier that RSV infection alone does not cause AHR in noninfected BALB/c mice [10]. Thus, prior allergen sensitization...
changes the AHR response to RSV infection. In this model, we infected mice 14 days after the last exposure of the mice to aerosolized OVA. From our previous work, we found that levels of type 2 cytokines in the lung supernatants are not detectable by ELISA 5 days after the last exposure to OVA aerosol [10]. Possible explanations for the effect that prior OVA sensitization has on RSV-induced AHR may include alteration in the distribution of lung dendritic cell populations, leading to altered antigen presentation and abnormal induction of T cell subsets; altered composition of memory CD4 T cells; or persistent, low-level production of type 2 cytokines in the lung.

Contrary to current dogma, in our studies, the increased AHR seen with RSV infection after allergen sensitization occurred in the setting of increased lung lymphocytes rather than eosinophilia. Thus, AHR associated with RSV infection and perhaps other viral infections is not strictly correlated with lung eosinophilia. It seems unlikely that eosinophil degranulation products were present at the time we measured the RSV-induced AHR, since the methacholine challenge measurements were made 2 weeks after RSV infection and 4 weeks after the last exposure to AHR, at times ≳ 1 week after lung eosinophils are undetectable by histopathologic analysis [13].

In this model, allergic sensitization before RSV protected against RSV-induced weight loss. This was a surprising finding, because these mice had increased RSV-induced AHR. The diminished weight loss seen in the OVA-RSV group was associated with decreased lung IFN-γ levels but with no change in viral replication or delay in viral clearance. We do not believe that the difference in IFN-γ levels explains the weight loss difference, because our group showed earlier that RSV infection caused weight loss both in mice lacking IFN-γ and in mice treated with anti–IFN-γ antibodies (authors’ unpublished data). Therefore, decreased lung IFN-γ levels may cause the increase in AHR seen with RSV infection after allergen sensitization but is not sufficient to explain the reduced weight loss.

In summary, we show in this model that RSV infection before allergic sensitization protects against the development of allergen-induced AHR and allergic airway inflammation, whereas RSV infection after allergic sensitization increases RSV-induced AHR. The AHR does not correlate with weight loss, lung eosinophilia, or changes in virus titer but correlates with a decrease in lung IFN-γ levels. Thus, the timing of RSV infection in relationship to allergic sensitization is critical in many clinically important features of RSV-induced disease, particularly AHR. Our data support the concepts that severe RSV-associated airway dysfunction occurs in a host with underlying allergic disease and that RSV does not directly induce the allergic phenotype.

References


