Respiratory Syncytial Virus (RSV)–Induced Airway Hyperresponsiveness in Allergically Sensitized Mice Is Inhibited by Live RSV and Exacerbated by Formalin-Inactivated RSV

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Respiratory syncytial virus (RSV)–induced disease is associated with recurrent episodes of wheezing in children, and an effective vaccine currently is not available. The use of 2 immunizations (a formalin-inactivated, alum-precipitated RSV vaccine [FI-RSV] given intramuscularly and live RSV given intranasally [LVIN]), with a control immunization, were compared in a well-characterized model of RSV challenge, with or without concomitant allergic sensitization with ovalbumin. FI-RSV caused a significant increase in airway hyperresponsiveness in mice after RSV infection during allergic sensitization, and this was associated with an increase in type 2 cytokine production. In contrast, immunization with LVIN did not change type 2 cytokine production and protected against RSV-induced airway hyperresponsiveness in the setting of allergic sensitization. This study suggests that immune modulation with RSV vaccination can have profound effects on RSV-induced airway disease and that prevention of airway hyperresponsiveness is an important end point in vaccine development.

Some viral infections cause or exacerbate wheezing-related illnesses in childhood. Up to 80%–85% of asthma exacerbations in school-age children are associated with viral respiratory tract infections [1], and severe attacks of asthma that lead to hospital admission are precipitated by viral infections [2]. In children <2 years old, respiratory syncytial virus (RSV) is the most common cause of acute bronchiolitis and wheezing [3]. In addition, severe RSV infection is associated with childhood asthma [4]. Preventing the airway-associated morbidity caused by RSV and other viral infections by immunization would have tremendous social and financial benefit. However, previous attempts at immunization against RSV in infants have been unsuccessful. A formalin-inactivated, alum-precipitated RSV vaccine (FI-RSV) developed in the late 1960s was administered intramuscularly (im) and caused an enhanced illness in a high percentage of vaccinees, who subsequently were infected with wild-type RSV [5]. This enhanced illness was characterized by typical features of severe wild-type RSV infection, including bronchiolitis, hypoxemia, and radiographic evidence of pneumonia; in addition, neutrophil and eosinophil infiltration, not usually associated with RSV-induced disease, was also present [5]. Clinical trials using parenteral vaccination with live RSV [6] or nasal infection with live RSV have not been protective, but neither produced enhanced illness after subsequent RSV infection.

Animal models provide tools to test the immunologic and physiologic impact of vaccines. We [7] and others [8, 9] have developed murine models of airway hyperresponsiveness (AHR) when RSV infection is combined with allergic airway sensitization. We found that RSV causes an increase in AHR only when infection occurs either during or after allergic sensitization with ovalbumin (OVA) and that the AHR persists >14 days after infection, a time after which AHR to allergen sensitization alone has markedly diminished [7]. We, therefore, attempted to abrogate the prolonged increased AHR caused by RSV infection in allergically sensitized animals by using a series of vaccines that specifically modulate the murine response to RSV. In this study, we tested FI-RSV given im and live RSV given intranasally (LVIN), administered 36 days before the onset of allergen sensitization and 72 days before RSV infection. To focus on the effect of vaccine-induced immunologic memory on RSV-induced AHR, we chose the length of time between vaccination and infection so that the acute inflammation and primary immune response to vaccination had resolved before allergen sensitization and infection.

Methods

Mice. Pathogen-free 8-week-old female BALB/c mice were purchased from Harlan (St. Louis). They were shipped in filtered
crates and housed in a high efficiency particulate arrestance-filtered, duo-flow laminar flow unit. Cages, bedding, food, and water were sterilized before use. Room temperature was maintained at 27°C, and a 12-h-on–12-h-off light cycle was provided.

**Study protocol.** There were 9 groups of mice (figure 1), all of which were vaccinated on day –72. Groups 1–3 were vaccinated with FI-RSV. Groups 4–6 were vaccinated with LVIN containing \(2.5 \times 10^6\) pfu of RSV. Groups 7–9 were given a mock vaccine, a formalin-inactivated preparation that did not contain RSV. The groups that were sensitized with OVA underwent intraperitoneal (ip) injection of OVA formulated with alum (day –16) and daily exposure to aerosolized OVA for 8 days (days –2–6), as more fully described below in the allergen sensitization protocol section. The groups that were not sensitized with OVA underwent a sham allergen–sensitization protocol, which is also described below.

Mice were infected with RSV or a control culture medium preparation on day 0. Just before infection, 3 mice in each group were harvested to analyze the histopathologic background in which infection took place. Lungs from 4 mice in each group were harvested for plaque-forming units on day 4, the day of peak viral replication [10], and for quantifying cytokine protein levels in the lung. In another study, we showed that cytokine protein levels in RSV-infected mice allergically sensitized with OVA (OVA/RSV mice) peak between days 3 and 5 [11]. On day 14, mice from each group underwent methacholine challenge, immediately followed by collection of serum by retroorbital puncture, sacrifice, and bronchoalveolar lavage.

**Cells and virus.** HEp-2 cells were maintained in Eagle MEM (EMEM) supplemented with glutamine, amphotericin, gentamicin, penicillin G, and 10% fetal bovine serum. The A2 strain of RSV was provided by Robert Chanock (National Institutes of Health, Bethesda, MD). Master stocks and working stocks of RSV were prepared as described elsewhere [10].

**Preparation of vaccine products.** RSV stocks were grown as above, quick frozen, and stored at –70°C. FI-RSV was made following the general procedure used to produce the vaccine used in earlier clinical trials [12]. Large volumes of RSV stock were incubated for 72 h at 37°C, with or without 4% wt/vol formalin phosphate. The stocks then were centrifuged (17,700 g) for 17 h. The formalin-inactivated pellet was resuspended in EMEM without serum (1/40 the original volume), and one-fourth was stored at 4°C in 1-mL aliquots. The remaining suspensions were diluted 4-fold, and 4 mg/mL aluminum phosphate was added. The buffered precipitate was centrifuged at 1000 g for 30 min, resuspended in 1/40 of the original virus stock volume of EMEM without serum, sonicated for 15 s, and stored at 4°C in 1-mL aliquots. A mock preparation, in which the HEp-2 cells were not infected with RSV, was made in an identical fashion.

**Allergen sensitization protocol.** Mice in the OVA group were injected ip with 0.1 mL (10 mg) of OVA (chicken OVA, grade V; Sigma, St. Louis) complexed with 2 mg of Al(OH)₃ on day 16, as described elsewhere [7]. On days –2–6, the mice were placed in an acrylic box and exposed to aerosols of 1% OVA diluted in sterile PBS, using a jet nebulizer (Ultraneb 99; DeVilbiss, Somerset, PA), for 40 min each day. Opposite the aerosol orifice was a small exhaust orifice vented into a chemical hood to ensure continuous air flow. Age-matched control animals received mock sensitization with ip Al(OH)₃, on day 16.

**Experimental Protocol**

![Experimental Protocol](https://example.com/image.png)

**Figure 1.** Time line of experimental protocol and definition of experimental groups. Group names are immunization status followed by ovalbumin (OVA) sensitization and respiratory syncytial virus (RSV) infection status (i.e., OVA, RSV, and OVA/RSV groups). Immunizations: FI-RSV, formalin-inactivated alum-precipitated RSV vaccine given intramuscularly; LVIN, live RSV given intranasally; mock, immunization with a formalin-inactivated alum-precipitated preparation in which no RSV was present. Allergen sensitization consisted of 1 intraperitoneal injection of aluminum hydroxide combined with OVA, followed 2 weeks later by 8 days of exposure to 1% OVA aerosol for 40 min each day. Sham sensitization consisted of 1 intraperitoneal injection of aluminum hydroxide and no exposure to OVA aerosol.
RSV, RSV-infected group. nasally (LVIN) or with the OVA-sensitized group that was given mock. RSV infection with this procedure causes bronchial inflammation were graded as follows: 0, no infiltrate; 1, minimal increased cellularity without widening of septa; 2+, obvious increased cellularity with widening of septa; and 3+, markedly increased cellularity with thickened septa (this score also includes blood or edema fluid in the tissue space).

Quantitation of interleukin (IL)-4, IL-5, IL-13, and interferon (IFN)-γ in lung tissues. Levels of IL-4, IL-5, IL-13, and IFN-γ in lung tissues of the 4 groups of mice were measured using commercially available ELISA kits (IL-4, IL-5, and IFN-γ from Endogen, Woburn, MA; IL-13 from R&D Systems, Minneapolis), according to the manufacturers’ protocols. One lung from each of 3 mice in each group was harvested on day 4 and analyzed separately for cytokine levels. In brief, the lung was harvested and

![Figure 2](https://academic.oup.com/jid/article-abstract/182/3/671/867353)
groups, compared with any other group. OV_A) group or the LVIN-immunized OV_A/RSV (LVIN-OV_A/RSV) group. The AHR of the mock-OV_A/RSV mice was significantly greater lung resistance than did all other groups (\(P < .05\)). At the final dose of methacholine, 3700 \(\mu g/\text{kg}\), the FI-RSV±immunized RSV-infected mice; and for all other groups. OVA, ovalbumin-sensitized group; LVIN, live RSV given intranasally. * for FI-RSV±immunized OV_A/RSV group and OV_A groups. OV_A, ovalbumin-sensitized group; LVIN, live RSV given intranasally. * for FI-RSV±immunized OV_A/RSV group and OV_A groups, compared with any other group.

quick-frozen in 2 mL of culture medium with serum. At the time of cytokine level determination, the lung and the culture medium in which the lung had been frozen were placed in a mortar. The lung was ground using a pestle and ground glass. The solution of the ground lung and the ground glass then was centrifuged at 1000 \(g\) for 10 min. The supernatant was added to precoated wells and incubated for 2 h. Dilutions of recombinant cytokine were included for generation of a standard curve. Peroxidase-labeled anti-cytokine antibody was added to detect bound cytokine, and the plates were developed by the addition of tetramethylbenzidine substrate. Concentrations of cytokines in the lung supernatants were calculated from the standard curve produced. The cytokine level from each lung was measured in duplicate.

**Statistical analysis.** Results are expressed as mean ± SE. Cellular composition of the bronchoalveolar lavage fluid and lung histopathologic results were compared with a Mann-Whitney \(U\) test for unpaired data. Dose-response curves to methacholine were compared by analysis of variance. Differences were considered to be significant if \(P < .05\).

**Results**

**Methacholine-induced airway responsiveness.** On day 14, AHR, as measured by increases in airway resistance to iv methacholine, was determined in all 9 groups of mice (figure 2). At the final dose of methacholine, 3700 \(\mu g/\text{kg}\), the FI-RSV±immunized OVA/RSV (FI-RSV±OVA/RSV) group had significantly greater lung resistance than did all other groups (\(P < .05\)), including the mock-immunized OVA/RSV (mock-OVA/RSV) group. The AHR of the mock-OVA/RSV mice was greater than that of either the mock-immunized OVA (mock-OVA) group or the LVIN-immunized OVA/RSV (LVIN-OVA/RSV) group. This signifies that RSV infection during allergen sensitization caused an increase in AHR at day 14, compared with allergen sensitization alone, and that the LVIN immunization was protective against the AHR that occurred with RSV infection in the setting of allergen sensitization.

There was no difference in AHR between the LVIN-immunized OVA (LVIN-OVA) and the mock-OVA groups, revealing that prior RSV infection at a dose of \(2.5 \times 10^6\) pfu neither protected nor exacerbated AHR to OVA when given at this time interval. In those mice that were allergen sensitized but not RSV infected, the FI-RSV±immunized OVA (FI-RSV±OVA) group had significantly greater AHR than did the mock-OVA group, which suggests a deleterious effect of FI-RSV on allergic airway disease.

**FI-RSV immunization increases type 2 cytokines, compared with mock immunization, whereas these cytokines decrease after LVIN.** The concentrations of IFN-\(\gamma\), IL-4, IL-5, and IL-13 in the lung supernatants were measured on day 4, by ELISA. The IL-4 concentrations on day 4 in the FI-RSV±OVA/RSV and FI-RSV±OVA groups were greater than those of any other group (figure 3). The FI-RSV±OVA/RSV group had the highest concentration of IL-5 on day 4 (figure 4). Thus, FI-RSV immunization before RSV infection during allergen sensitization caused a marked induction of type 2 cytokines. This is in distinct contrast to mock immunization, in which the mock-OVA group had significantly higher levels of IL-5 and a moderate increase in IL-4 (\(P = .07\)), compared with the mock-OVA/RSV group.

The mock-OVA group had an increase in IL-4 on day 4, compared with the mock-OVA/RSV group.

**Figure 3.** Concentrations of interleukin (IL)-4 in lung supernatants on day 4 after challenge. \(n = 3\) for respiratory syncytial virus (RSV)-infected mice sensitized with ovalbumin (OVA/RSV) and immunized with formalin-inactivated alum-precipitated RSV vaccine (FI-RSV); \(n = 3\) for RSV-infected mice intramuscularly injected with a formalin-inactivated preparation that did not contain RSV (mock) and for FI-RSV-immunized RSV-infected mice; and \(n = 4\) for all other groups. OVA, ovalbumin-sensitized group; LVIN, live RSV given intranasally. * for FI-RSV±immunized OVA/RSV group and OVA groups, compared with any other group.

**Figure 4.** Concentrations of interleukin (IL)-5 in lung supernatants from mice on day 4 after challenge. \(n = 3\) for respiratory syncytial virus (RSV)-infected mice sensitized with ovalbumin (OVA/RSV) and immunized with formalin-inactivated alum-precipitated RSV vaccine (FI-RSV); \(n = 3\) for RSV-infected mice intramuscularly injected with a formalin-inactivated preparation that did not contain RSV (mock) and for FI-RSV-immunized RSV-infected mice; and \(n = 4\) for all other groups. *\(P < .05\) for FI-RSV±immunized OVA/RSV group, compared with any other group. OVA, ovalbumin-sensitized group; LVIN, live RSV given intranasally. †\(P < .05\) for mock-immunized OVA mice, compared with the mock-immunized OVA/RSV group.
Figure 5. Concentrations of interleukin (IL)-13 in lung supernatants from mice on day 4 after challenge. \( n = 3 \) for respiratory syncytial virus (RSV)–infected mice sensitized with ovalbumin (OVA/RSV) and immunized with formalin-inactivated alum-precipitated RSV vaccine (FI-RSV); \( n = 3 \) for RSV-infected mice intramuscularly injected with a formalin-inactivated preparation that did not contain RSV (mock) and for FI-RSV–immunized RSV-infected mice; and \( n = 4 \) for all other groups. OVA, ovalbumin-sensitized group; LVIN, live RSV given intranasally. * for FI-RSV–immunized OVA/RSV and OVA mice, compared with any other group.

Figure 6. Concentrations of interferon (IFN)–\( \gamma \) in lung supernatants from mice on day 4 after challenge. \( n = 3 \) for respiratory syncytial virus (RSV)–infected mice sensitized with ovalbumin (OVA/RSV) and immunized with formalin-inactivated alum-precipitated RSV vaccine (FI-RSV); \( n = 3 \) for RSV-infected mice intramuscularly injected with a formalin-inactivated preparation that did not contain RSV (mock) and for FI-RSV–immunized RSV-infected mice; and \( n = 4 \) for all other groups. OVA, ovalbumin-sensitized group; LVIN, live RSV given intranasally. * for FI-RSV–immunized OVA/RSV and FI-RSV–OV A/RSV groups, compared with any other group.

Discussion

We showed elsewhere that RSV infection occurring during allergen sensitization with OVA causes a prolongation in AHR for \( \leq 2 \) weeks after infection, whereas infection with RSV in the absence of allergic sensitization does not affect AHR [7]. We sought to determine if this increased AHR seen with RSV infection in the setting of allergic inflammation could be abrogated by immunization. These studies demonstrate that immunization with LVIN reduces AHR caused by RSV infection during OVA sensitization, whereas FI-RSV administered exacerbated AHR. This increase in AHR in the FI-RSV–OVA/RSV group was accompanied by an increase in IL-5 in lung supernatants (compared with those in the FI-RSV–OVA group), whereas there was no difference in the IL-4 and RSV (mock-RSV) groups was 6.12 ± 0.18 and 6.15 ± 0.36 log\(_{10}\) pfu/g, respectively, which were similar to values we reported elsewhere, using similar conditions, for primary RSV infection [10]. Although there were no differences in the log\(_{10}\) pfu/g between the mock-OVA/RSV and mock-RSV groups on day 4, viral replication was inhibited in the FI-RSV– and LVIN-immunized groups (\( P < .05 \)). The RSV titers in the FI-RSV–immunized RSV (FI-RSV–RSV; 5.28 ± 0.72 log\(_{10}\) pfu/g) and FI-RSV–OVA/RSV (4.48 ± 0.11 log\(_{10}\) pfu/g) groups were both greater (\( P < .05 \)) than those in the LVIN-immunized RSV (LVIN-RSV) and LVIN-OVA/RSV groups, in which titers were undetectable.
IL-13 levels in these 2 groups. In contrast, the mock-OVA group had significantly greater levels of IL-5 than did the mock-OVA/RSV mice.

Taken together, these results indicate that FI-RSV fundamentally changes the immune response in RSV-infected, OVA-sensitized mice and that FI-RSV preferentially induces IL-5 production in the lungs of OVA/RSV mice. FI-RSV also changed the physiologic and immunologic responses to OVA sensitization in the absence of RSV infection. The FI-RSV-OVA group of mice had increased AHR, compared with the mock-OVA group, and this paralleled an increase in IL-5 and IL-13 concentrations in the FI-RSV-OVA group. IL-5 is a critical cytokine for eosinophil differentiation and survival and has been implicated as a critical factor in the induction of AHR [13].

In contrast, the IL-4 and IL-5 levels were similar in the mock-OVA/RSV and LVIN-OVA/RSV groups, although LVIN significantly abrogated AHR, which suggests that the protective effect of the LVIN vaccine was independent of acute type 2 cytokine production and was, most likely, a result of RSV-specific immune modulation. We did find that the mock-OVA mice had a slight nonsignificant increase in IL-4, a significant increase in IL-5, and no change in IL-13 levels in the day 4 lung supernatants, compared with the levels for the LVIN-OVA group; however, there was no difference in AHR between the 2 groups at day 14. This suggests that a live RSV vaccine neither definitively up- nor down-regulates allergen sensitization, a critical issue in considering vaccine side effects.

The protection afforded the OVA/RSV group by LVIN immunization (LVIN-OVA/RSV group) was associated with a decrease in viral replication. FI-RSV–OVA/RSV mice also had a decrease in virus titers but had significantly greater AHR than did mock-OVA/RSV mice. Mock-RSV mice had high virus titers but no AHR, so virus titer did not correlate with AHR. Neither the FI-RSV nor LVIN vaccines altered the histology of the airways or lung parenchyma at the time of infection in the allergically sensitized mice, which suggests that vaccination did not affect quantitatively cellular allergic inflammation and therefore, by inference, might modify the composition of the memory T cell response to RSV infection.

The protective effect of LVIN immunization on AHR in the OVA/RSV mice also could not be clearly explained by an alteration in the type 2 cytokine profile, because there was no difference in the levels of IL-4, IL-5, or IL-13 in the lung supernatants of LVIN-OVA/RSV and mock-OVA/RSV groups. In another study, we found that the type 1 and type 2 cytokine profiles in this model peak between days 3 and 5 [11], and in this experiment we measured cytokines at day 4. In prior work, there was an increase in AHR in nonvaccinated mice that had been RSV infected during OVA sensitization, compared with mice that were only OVA sensitized, although the type 2 cytokines were markedly increased in the mice sensitized with OVA alone, compared with the OVA sensitized and RSV-infected mice. The cytokine profile associated with prolonged AHR in OVA-sensitized, RSV-infected mice was shown to include both type 1 and 2 cytokines, whereas only type 2 cytokines were detected in allergically sensitized, noninfected mice with more-transient AHR [11].

In the present study, we found that AHR correlated most closely with IFN-γ levels in the 3 groups of mice that were sensitized with OVA and infected with RSV (OVA/RSV groups). The FI-RSV–OVA/RSV group had the highest levels of IFN-γ, the LVIN-OVA/RSV group had the lowest level of IFN-γ, and the mock-OVA/RSV group had an intermediate level. In those mice that were sensitized with OVA but not infected with RSV, AHR correlated with the levels of type 2 cytokines in the lung supernatants. These results suggest that the timing and composition of virus-stimulated and allergically induced inflammation are critical determinants of virus-associated AHR.

Virus replication did not correlate with AHR in the combined OVA/RSV challenge. Although FI-RSV decreased RSV replication and increased AHR, live virus immunization completely suppressed virus replication after subsequent RSV challenge, yet reduced AHR. Since LVIN protected against subsequent RSV infection, a better test of this concept may have been partial immunization with a lower dose of live virus or a recombinant vector as immunogen. This represents a limitation of the use of live virus in the murine system. However, there was a significant increase in IFN-γ in the LVIN-OVA/RSV group, compared with the LVIN-OVA group, which indicated that the RSV challenge was immunogenic, even in the setting of prior LVIN immunization. Also, the FI-RSV–immunized mice had greater AHR, but less RSV replication, than did mock-immunized mice. These findings suggest that the composition of the immune response was a more important determinant of AHR than was the level of RSV replication.
In summary, we have shown that our model of allergic sensitization and RSV infection may be used to differentiate the effects of vaccination on virus-associated AHR. One vaccine, LVIN, ablates RSV-induced AHR even in the setting of allergic inflammation, whereas, in contrast, FI-RSV exacerbates AHR. These differences in effect on AHR suggest that prevention of AHR should be an important end point of RSV vaccine development. Our study indicates that effective RSV immunization may decrease the incidence of childhood asthma, as well as prevent RSV infection.

References