MAJOR ARTICLE

Timing of Infection and Prior Immunization with Respiratory Syncytial Virus (RSV) in RSV-Enhanced Allergic Inflammation

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Respiratory syncytial virus (RSV) infection has been shown to be a risk factor for the development of allergy in humans and mice. The allergy-enhancing properties of RSV may be dependent on atopic background and an individual's history of RSV infection. We examined the influence of the timing of infection and prior inoculation with RSV in a mouse model of allergic asthma. Mice were sensitized to and challenged with ovalbumin (OVA) and were inoculated with RSV either before or during the sensitization or challenge period. One group of mice was inoculated with RSV both before sensitization to OVA and during challenge with OVA. Increased pulmonary expression of interleukin (IL)–4, IL-5, and IL-13 mRNA and aggravated alveolitis and hypertrophy of mucus-producing cells were observed only when OVA-sensitized mice were inoculated with RSV shortly before or during challenge with OVA. Despite protection against viral replication, prior inoculation with RSV did not abrogate RSV-enhanced, OVA-induced expression of T helper 2 (Th2) cytokines in the lung. In conclusion, inoculation with RSV enhances allergic disease only when the immune system has already been Th2-primed by the allergen (i.e., OVA). This RSV-enhanced allergy is not completely abrogated by prior inoculation with RSV.

Respiratory syncytial virus (RSV), a member of the family *Paramyxoviridae*, genus *Pneumovirinae*, is the most common cause of respiratory-tract infections in young children [1]. The clinical presentation can vary, from mild upper respiratory–tract illness to severe bronchiolitis and pneumonia [2]. Epidemiological data show that almost all children become infected with RSV during their first or second year of life and that the incidence of RSV bronchiolitis reaches a maximum at the age of 2 months [3]. Because severe RSV infection induces asthmalike symptoms (e.g., wheezing and airway hyperreactivity [AHR]), studies have been performed to investigate the effect of RSV bronchiolitis on the development of respiratory allergy and asthma in children. Thus far, controversial results have been reported [4–9]. As a result, the precise role of RSV infection in the development of allergic disease in children is not clear.

Allergic asthma is a Th2-associated airway inflammation, characterized by the infiltration of eosinophils and the production of interleukin (IL)–4, IL-5, and IL-13 by T lymphocytes in response to certain antigens [10, 11]. In contrast, RSV infection predominantly induces a Th1 immune response, which is characterized by the production of IL-12 and interferon (IFN)–γ [12, 13]. We [14] and other researchers [15] have previously demonstrated in a mouse model of allergic asthma that RSV infection, when induced during the provocation phase of exposure to allergens, aggravates the consequences of airway sensitization to and challenge with ovalbumin (OVA), resulting in enhanced pulmonary expression of Th2 cytokines, allergy-associated lung pathologic lesions, and AHR. In addition, the RSV-induced Th1 response remains unchanged [14].

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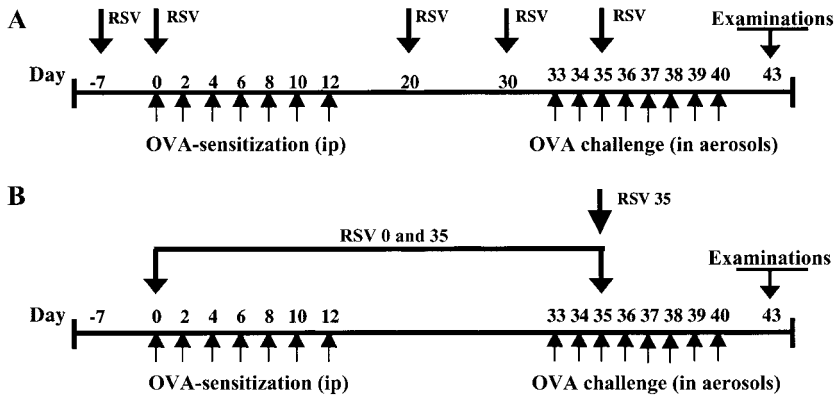

Figure 1. Study design. *A,* To investigate the influence of the timing of inoculation with respiratory syncytial virus (RSV) on RSV-enhanced ovalbumin (OVA) allergy, OVA-sensitized and -challenged BALB/c mice received 10^7 pfu of RSV on day 0, 7, 20, 30, or 35 *(arrows). B,* To examine the influence of prior inoculation with RSV on RSV-enhanced OVA allergy, OVA-sensitized and -challenged mice received 1.5×10^7 pfu of RSV on days 0 and 35. inl, Intranasal; ip, intraperitoneal.

The results of several studies have suggested that the time between infection and exposure to allergens may be critical in determining whether viral [16, 17] or bacterial [18] infection will influence the development of respiratory allergy in mice. To gain more insight into the mechanisms of RSV-enhanced OVA allergy, we investigated whether the timing of inoculation with RSV (i.e., before or during the period of sensitization to and challenge with the allergen) determines the outcome of allergic disease in mice.

As mentioned above, most children become infected with RSV during their first or second year of life [3]. Because immunity against RSV is only partial, reinfections can occur throughout life [2]. This means that an RSV infection that occurs shortly before or during challenge with an allergen might have been preceded by prior RSV infections (i.e., before sensitization to an allergen). The individual's history of RSV infection could modulate viral replication and the immune response during a second RSV infection, thus influencing its potential effect on respiratory allergy. Whether prior infection with RSV enhances or protects against RSV-enhanced OVA allergy needs to be investigated. In the present study, we therefore inoculated mice with RSV before sensitization to and challenge with OVA and reinfected them during the challenge with OVA. The cytokine and inflammatory data obtained from the experiments presented here lead to a better understanding of the mechanism by which RSV infection affects respiratory allergy.

MATERIALS AND METHODS

Virus. Human RSV stock A2 (RSV A2) was obtained from the American Type Culture Collection (ATCC). The virus was cultured on HEp-2 cells (ATCC) in RPMI 1640 medium (Gibco BRL, Life Technologies) containing 10% heat-inactivated fetal calf serum (Greiner), 2 mmol/L glutamine, 100 IE/mL penicillin, and 100 U/mL streptomycin, as described elsewhere [19]. The virus inoculum contained 3×10^8 pfu of RSV/mL, as assessed by use of a quantitative plaque-forming assay [19].

Mice. Female BALB/c mice were obtained from Harlan Olac and were used at 6–10 weeks of age. One week before the experiments started, mice belonging to 1 treatment group ($n = 6$) were housed together in 1 cage, according to the experimental set-up in a temperature-controlled animal room. Mice were kept in a 12-h light/dark cycle in pathogen-free conditions and received food and water ad libitum. The committee on animal welfare of the Dutch National Institute of Public Health and the Environment approved the study.

Experimental design. Mice were sensitized to OVA by intraperitoneal (ip) administration of 0.5 mL of OVA (Grade II; Sigma-Aldrich) (20 μg/mL OVA in saline) for 7 times on every other day starting at day 0. From day 33 through day 40, mice were challenged with aerosol OVA (2 mg/mL of saline) for 5 min on 8 consecutive days [14, 20].

To examine the influence of the time point of inoculation with RSV during the sensitization/challenge protocol, OVA-sensitized mice were inoculated intranasally (inl) with RSV (1.5×10^7 pfu in 50 μL) at different time points, according to the schedule shown in figure 1*A*. The infected mice were compared with OVA-allergic mice inoculated with uninfected culture lysate (mock) at the same time point. To investigate the influence of prior inoculation with RSV on RSV-enhanced allergy, 1 group received 1.5×10^7 pfu of RSV on both day 0 and day 35 of the experimental protocol (figure 1*B*). Before inoculation, mice were anesthetized with halothane. RSV-enhanced lesions are best analyzed at day 43 of the experimental protocol (day 3 after the last challenge with OVA) [14]; therefore, on that day, mice were anesthetized ip with Ketamine Rompun (xy-

lazine; Bayer Benelux) Atropine and killed. Blood was obtained via orbital puncture. The lungs were removed. The right lung was snap-frozen in liquid nitrogen and stored until processed further. The left lung was intratracheally fixed with formalin and immersed in fixative.

Histopathologic examination. Formalin-fixed lungs were embedded in paraplast (Monoject). Transverse sections of 5 μm were stained with hematoxylin-eosin. In a blinded fashion, an independent observer examined the slides for peribronchiolitis (i.e., infiltration of inflammatory cells in the peribronchiolar space), alveolitis (i.e., infiltration of inflammatory cells in the alveolar wall), perivasculitis (i.e., infiltration of inflammatory cells in the perivascular space), hypertrophy of mucus-producing glands, and eosinophilia. Lung lesions were scored semiquantitatively as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5).

Isolation of RNA and reverse-transcriptase (RT) polymerase chain reaction (PCR). Total cellular RNA was isolated from frozen lung samples by use of Trizol Reagent (GibcoBRL, Life Technologies). Ten microliters of total lung RNA was reverse transcribed, and cytokine PCRs (IL-4, IL-5, IL-13, IFN-γ, and IL-12) of the cDNA were performed as described elsewhere [12]. Eotaxin RT-PCR was performed according to the same protocol as that used for the cytokine RT-PCRs, using sense (5′-CCCCAACACACTACTGAAGA-3′) and antisense (5′-CTACATGAAGCCAAGTCCTT-3′) primers. To prevent interassay variation, PCRs of all samples from 1 experiment were done simultaneously and were loaded on the same agarose gel for quantification. Band intensities were analyzed by use of the Molecular Analyst software system (Bio-Rad Laboratories). All results were corrected for mRNA content of the sample by use of β-actin mRNA as a standard. β-Actin mRNA was amplified by use of the mouse control amplifier set (Clontech), according to the manufacturer's protocol. For quantification of the RSV load in lung tissue, RT-PCR was performed with RSV F-protein–specific primers (upper primer, 5′-TTAACCAGCAAAGTGTTAGA-3′; lower primer, 5′-TTTGTTATAGGCATATCATTG-3′), as described elsewhere by Paton et al. [21]. The PCR product was analyzed by detecting the band intensities on the agarose gel by use of the Molecular Analyst software system (Bio-Rad Laboratories).

IgE antibodies in serum. Total and OVA-specific IgE antibodies in serum were determined by use of a modified capture ELISA, according to the protocol of van Halteren et al. [22]. As coating antibody, rat anti–mouse IgE monoclonal antibody EM-95 was used [23]. Total IgE was detected by use of biotinylated rat anti–mouse IgE (Pharmingen). Total concentrations of IgE were calculated by interpolation from a standard titration curve with known concentrations of recombinant mouse IgE (Pharmingen). The detection limit of the total IgE assay was 900 pg/mL. OVA IgE was detected with digoxigenin-coupled OVA. OVA-specific IgE levels were expressed as the optical density at 490 nm (OD_{490}) of a 1:32 diluted serum.

Statistical analysis. Data are presented as mean ± SEM. Statistical significance was determined for differences in cytokine ratios and IgE concentrations by use of the Student's *t* test (Excel; Microsoft) and for differences in histological scores by use of the nonparametric Wilcoxon test (SAS; SAS Institute).

RESULTS

Influence of RSV inoculation time point on respiratory allergy. We previously observed that inoculation with RSV, when performed during challenge with OVA, enhances OVA-induced pulmonary infiltration of inflammatory cells and expression of IL-4, IL-5, and IL-13 mRNA [14]. In the present study, we examined whether the timing of inoculation during sensitization to and challenge with the allergen is critical. Mice were therefore inoculated with RSV before or during sensitization to OVA and before or during challenge with OVA (figure 1*A*).

As shown in figure 2, RSV enhanced the expression of IL-4 and IL-13 mRNA in lung tissue of OVA-allergic mice only when inoculation was performed shortly before (day 30) or during (day 35) challenge with OVA, compared with OVA/mock-treated control mice. In addition, inoculation with RSV at all 3 time points after sensitization to OVA (days 20, 30, and 35) enhanced pulmonary expression of IL-5 mRNA in OVA-allergic mice. Simultaneous with the enhanced expression of Th2 cytokine mRNA, inoculation with RSV after sensitization to OVA (day 20, 30, or 35) induced enhanced expression of IFN-γ and IL-12 mRNA in OVA-allergic mice, compared with OVA/mock-treated control mice (figure 2). In addition, pulmonary expression of IL-4, IL-13, and IFN-γ mRNA was significantly higher in allergic mice inoculated on day 30 or 35, compared with the other time points of inoculation with RSV.

In parallel with the increased expression of Th2 cytokine mRNA, RSV enhanced hypertrophy of mucus-producing epithelial cells only when inoculation was performed shortly before (day 30) or during (day 35) challenge with OVA, compared with OVA/mock-treated control mice. Likewise, the alveolar inflammation was significantly increased, compared with OVA/mock-treated control mice, only when inoculation with RSV was performed on day 20, 30, or 35. In addition, alveolitis and hypertrophy of mucus-producing epithelial cells were significantly enhanced in lungs of allergic mice inoculated with RSV on day 35, compared with the other time points of inoculation with RSV. In contrast, no relationship between increased levels of Th2 cytokines and peribronchiolar and perivascular inflammation was observed. Finally, pulmonary eosinophilic infiltration could only be observed in OVA-allergic mice inoculated with RSV on day 35. However, there was no difference between the OVA/mock- and OVA/RSV-treated mice, indicating that

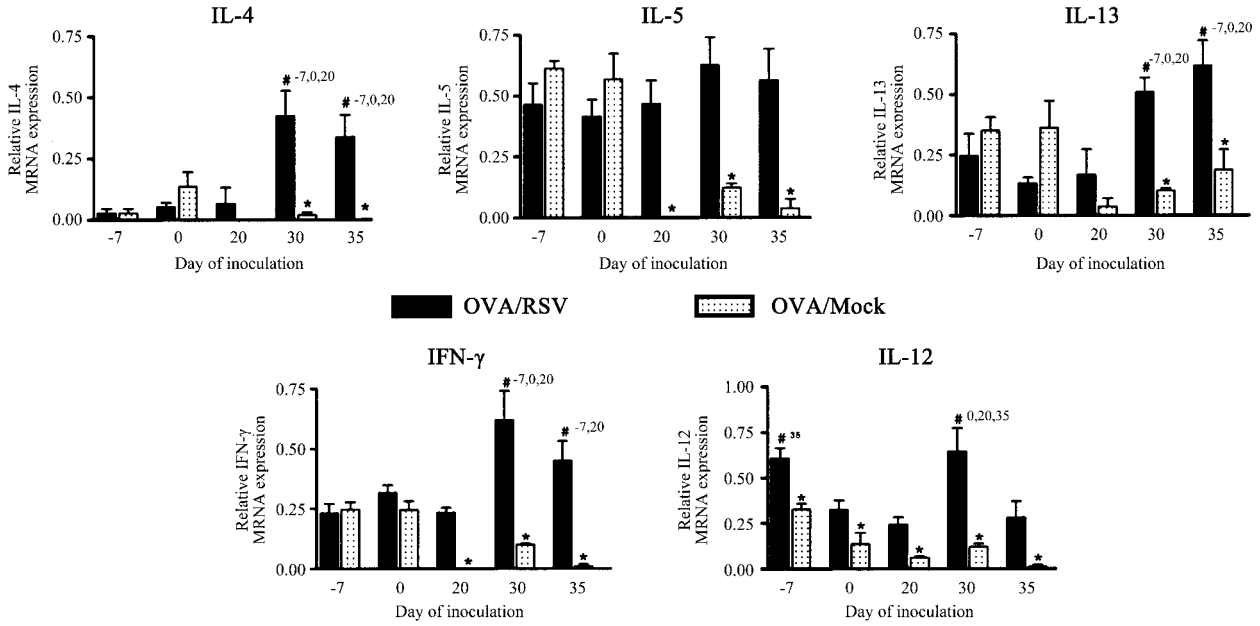

Figure 2. Relative expression against β-actin of lung interleukin (IL)–4, IL-5, IL-13, interferon (IFN)–γ, and IL-12 mRNA, determined at day 43 of the experimental protocol (i.e., day 3 after the last challenge with ovalbumin [OVA]). Mice were inoculated with respiratory syncytial virus (RSV) at different time points during sensitization to and challenge with OVA *(black bars)*. Control mice were inoculated with mock virus at the corresponding time points *(hatched bars)*. Mean values ± SEM (n = 6/group) are depicted. *P < .05, OVA/RSV- vs. OVA/mock-treated mice. #P < .05, OVA-treated mice inoculated with RSV at different time points.

inoculation with RSV did not affect OVA-induced eosinophilia in the present study. The lung pathologic lesions of OVA-sensitized and -challenged mice inoculated with RSV on day 35 is illustrated in figure 3.

To further elucidate the influence of RSV infection on OVA allergy, total and OVA-specific IgE levels were determined in serum at day 43 of the experimental protocol. Sensitization to and challenge with OVA resulted in the production of OVA-specific IgE in serum. As demonstrated elsewhere [14], inoculation with RSV during challenge with OVA did not increase the production of total and OVA-specific IgE, compared with the corresponding OVA/mock-treated mice (data not shown). However, inoculation with RSV on day 35 was associated with significantly higher levels of both total (2.10 ± 0.45 μg/mL) and OVA-specific IgE (OD_{490} of 1.284 ± 0.092), compared with inoculation on day 0 (total IgE, 0.66 ± 0.12 μg/mL; OVA-specific IgE, OD_{450} of 0.698 ± 0.176) or day 30 (total IgE, 0.77 ± 0.21 μg/mL; OVA- specific IgE, OD_{450} of 0.559 ± 0.134). However, the same dependence on time was observed after mock inoculation (data not shown).

Influence of prior inoculation with RSV on RSV-enhanced OVA allergy. We subsequently examined whether prior inoculation with RSV could diminish the effects of RSV on OVA allergy. We therefore compared the cytokine and inflammatory responses of OVA-allergic mice inoculated on day 35 (during challenge with OVA) with those of identically treated mice inoculated inl with live RSV on day 0 of the experimental protocol (figure 1*B*). To examine whether prior inoculation with RSV influences viral replication after reinfection, the RSV load in lung tissue was examined, by use of RT-PCR, at day 43 of the experimental protocol (i.e., day 8 after inoculation). We observed that prior inoculation with RSV (on day 0) significantly decreased the virus load. The virus load was 8 times lower in previously inoculated mice, indicating that prior inoculation with RSV diminished viral replication after reinfection (data not shown).

As demonstrated above, inoculation with RSV during challenge with an allergen enhanced the expression of the Th2 cytokines in lungs of OVA-allergic mice (figure 2). However, prior inoculation with RSV did not abrogate this effect (figure 4, *left*). In addition, prior inoculation with RSV did not significantly diminish the pulmonary expression of IFN-γ and IL-12 mRNA in OVA/RSV-treated mice (figure 4, *left*).

In contrast, prior inoculation with RSV diminished the alveolitis (from marked to minimal) and hypertrophy of mucus-producing epithelial cells (from marked to moderate) in lungs of OVA/RSV-treated mice (figure 4, *right*). However, peribronchiolar and perivascular inflammation in OVA/RSV-treated mice was not influenced by prior inoculation with RSV (figure 4, *right*). In addition, no significant reduction of the eosinophilic infiltration was observed (figure 4, *right*).

As mentioned above, no change in levels of total or OVA-specific IgE was measured in OVA-allergic mice inoculated with RSV on day 35, compared with control mice. Nevertheless,

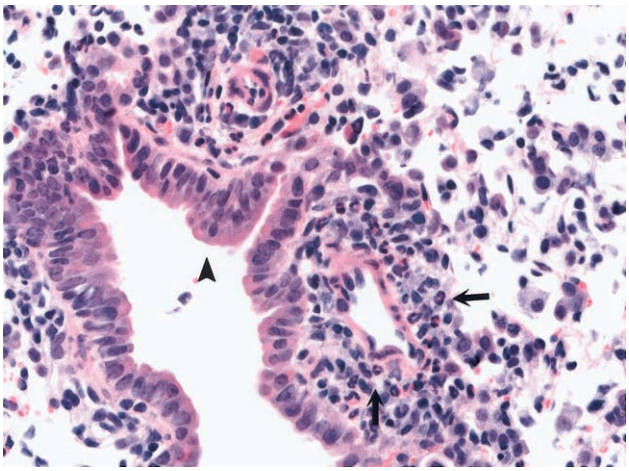

Figure 3. Lung section of ovalbumin (OVA)–sensitized and -challenged mouse inoculated with respiratory syncytial virus (RSV) on day 35. Lung pathologic lesions were analyzed at day 43 of the experimental protocol (i.e., day 3 after the last challenge with OVA). A marked hypertrophy of mucus-producing cells in bronchiolar epithelium was observed *(arrowhead),* together with perivascular infiltration of eosinophils *(arrows)* and lymphocytes (original magnification, ×1200).

prior inoculation with RSV decreased the concentration of both total ($P < .05$) and OVA-specific IgE ($P < .001$) in serum of OVA/RSV-treated mice, to approximately one-third of the concentration in serum of OVA/RSV-treated mice that were not previously inoculated with RSV (data not shown).

DISCUSSION

Results from different epidemiological studies in which the association between RSV infection in infancy and the development of respiratory allergy later in life was investigated have been inconclusive [4, 7]. In addition to epidemiological studies, studies using mouse models have investigated the mechanisms through which RSV infection influences the allergic immune response. We previously demonstrated, in a mouse model of allergic asthma, that RSV infection exacerbates the allergic Th2 cytokine response and lung pathologic lesions [14], whereas RSV infection in nonallergic mice did not induce a significant pulmonary Th2 cytokine response [12, 14]. In the present study, we have shown that the timing of RSV infection is critical for RSV-enhanced allergic responses. RSV infection enhanced the pulmonary Th2 cytokine response only when mice were inoculated after sensitization to OVA. The increased Th2 cytokine mRNA responses coincided with increased alveolitis and hypertrophy of mucus-producing epithelial cells. Since both alveolitis and pulmonary expression of IFN-γ mRNA are specifically enhanced by RSV infection [12], the enhanced alveolitis is likely a result of the effect of RSV alone, not RSV-enhanced allergy. Therefore, only the hypertrophy of mucus-producing cells seems to be related to increases in expression of Th2 cytokines. In contrast to the other parameters of allergic inflammation, perivasculitis and peribronchiolitis were not dependent on timing of inoculation with RSV, indicating that they were likely not correlated with elevated levels of pulmonary expression of Th2 cytokine mRNA. Because IL-5 has been identified as an eosinophil attractant [24, 25], and, because we observed high levels of expression of IL-5 mRNA in OVA-treated mice inoculated with RSV on day 35, we expected to see increased eosinophilia. A possible explanation for the absence of aggravated eosinophilia could be that the moment of examination was too late and that eosinophilia was already in decline [26]. Furthermore, Hessel et al. observed, in an identical mouse model of allergic asthma, that AHR (one of the key characteristics of asthma) can develop without the presence of eosinophils in the lung [20, 27]. We did not measure hyperreactivity of the airways, but we did observe a moderate hypertrophy of mucus-producing glands in allergic mice, which was enhanced even after inoculation with RSV. Increased production of mucus in the airways is reported to contribute to the development of AHR [28].

In contrast to the observation that inoculation with RSV before sensitization to and challenge with OVA did not influence the allergic responses in lung tissue, Peebles et al. demonstrated decreased AHR and production of pulmonary IL-13 protein in mice receiving a comparable treatment [29]. Since Peebles et al. also found that AHR and production of Th2 cytokines are not always correlated, these different observations do not have to be in conflict [30]. It is possible that factors other than Th2 cytokines could explain such observations.

A possible explanation for the time-dependent effect of RSV infection on OVA allergy, as observed in the present study, may be the OVA-induced cytokine milieu. Among antigen-presenting cells, dendritic cells (DCs) play a central role in regulating the cellular immune response and T cell polarization [31]. The outcome of T cell polarization by DCs can be either Th1 or Th2, depending on the microenvironment and the character of pathogen-induced inflammatory reactions [32]. In the model of sensitization to and challenge with OVA, a Th2 cytokine environment is induced by sensitization to OVA of lung tissue of mice. In this environment, presentation of RSV by DCs to T cells might stimulate T cells to become Th2 cells, aggravating the Th2 response. Coyle et al. [33] demonstrated in vivo (mice) and in vitro that production of IL-4 by OVA-specific $CD4^+$ T cells switches the virus-specific $CD8^+$ T cells in the lung to produce IL-5. These results indicate that the virus-specific immune response could be modified by a local, allergen-induced Th2 immune response. When inoculation with RSV is performed before or during sensitization to the allergen, no Th2

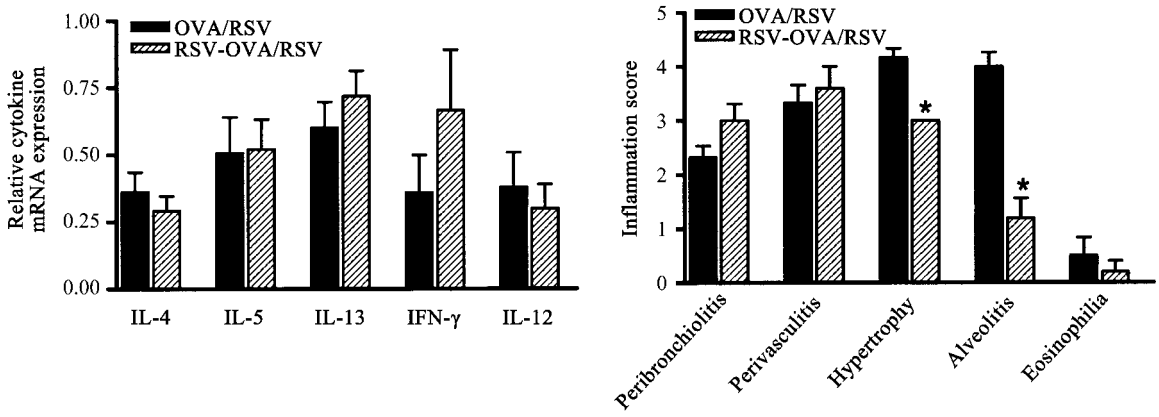

Figure 4. Relative expression against β-actin of lung interleukin (IL)–4, IL-5, IL-13, interferon (IFN)–γ, and IL-12 mRNA *(left)* and severity scores of pulmonary lesions *(right),* examined at day 43 of the experimental protocol (i.e., day 3 after the last challenge with ovalbumin [OVA]). OVA-sensitized and -challenged mice were inoculated with respiratory syncytial virus (RSV) on day 35 of the OVA-challenge period and were *(hatched bars)* or were not *(black bars)* inoculated with RSV on day 0 of the experimental protocol. Indicated pulmonary lesions are scored from absent (0) to severe (5). Mean values ± SEM (n = 6/group) are depicted. Statistical differences between differently treated groups are analyzed by use of the Students t test for mRNA expression data or the nonparametric Wilcoxon's test for histopathologic scores. *$P < .05$.

cytokine environment is present yet, which might result in failure to enhance the Th2 response.

In addition to the influence of the time of inoculation on the allergic inflammation, a high background for expression of IL-5 and IL-13 mRNA was observed in the lungs of mice inoculated with mock or RSV before sensitization to OVA. The reason for this high background is unclear. Nevertheless, because the RSV and mock inocula differ only in RSV content (since there is no RSV in the mock inoculum) significant differences between the RSV and mock groups must be specifically induced by RSV.

Epidemiological data show that almost all children become infected with RSV during the first or second year of life [3] and that reinfections with RSV occur throughout life. This phenomenon means that an RSV infection shortly before or during challenge with OVA might have been preceded by an earlier RSV infection before sensitization to the allergen. We have demonstrated that prior inoculation with RSV did not change the pulmonary expression of cytokine mRNA in allergic mice inoculated with RSV during challenge with OVA. Like expression of Th2 cytokine mRNA, peribronchiolar and perivascular inflammation and eosinophilia are not altered by prior inoculation with RSV. These results are in accordance with those of Peebles et al., who observed that inoculation with RSV before sensitization to the allergen has no effect on levels of IL-4 and IL-5 protein in the lungs of OVA/RSV-treated mice [34]. However, they found that inoculation with RSV protected against RSV-enhanced AHR. In the present study, prior inoculation with RSV diminished hypertrophy of mucus-producing cells in OVA-allergic mice infected with RSV. Taken together, these results suggest that increased hypertrophy of mucus-producing cells is related to enhanced AHR. In line with the results of previous studies [12, 34], prior inoculation with RSV dramatically diminished the virus load after secondary infection, indicating protection against viral replication. Prior inoculation with RSV also diminished alveolitis after secondary infection. This decreased alveolitis is probably due to the absence of RSV replication after prior inoculation, since alveolitis is specifically induced by RSV infection [12]. It is still unclear which factors are responsible for RSV-enhanced allergy. However, the lack of correlation between virus load and enhancement of allergy indicates that the antiviral inflammatory response, rather than virus-induced lesions, is responsible for allergy enhancement.

In summary, the timing of inoculation with RSV during sensitization to and challenge with OVA is critical for the development of allergic responses in mice. Increased pulmonary expression of Th2 cytokine mRNA and hypertrophy of mucus-producing cells were observed only when mice were inoculated with RSV after sensitization to OVA. Despite strongly reduced viral replication, previous inoculation with RSV could not abrogate the RSV-enhanced, OVA-induced expression of Th2 cytokines in the lungs. These data provide evidence that a preexisting Th2 cytokine milieu leads to RSV-enhanced respiratory allergy. In addition, prior inoculation with RSV is only partially beneficial for later RSV-enhanced respiratory allergy.

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