

The Role of Virus-specific Immunoglobulin E in Airway Hyperresponsiveness

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Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis during infancy and is associated with subsequent wheezing and asthma, but the nature of this association is not fully understood. We investigated the role of RSV-specific IgE antibodies in the pathophysiology of virus-induced airway dysfunction in a mouse model. Lung infection with RSV resulted in significant increases in mRNA expression for IgE and both of its high- and low-affinity receptors. In serum, virus-specific IgE antibodies reached peak levels by Day 21 after infection. Data from *in vitro* experiments show that RSV can induce mast cell degranulation, but only if these cells are sensitized with specific IgE. When passively sensitized *in vivo* with virus-specific IgE, mice developed exaggerated airway responsiveness to methacholine on airway infection, an effect that required the high-affinity receptor of IgE. These data suggest that RSV-specific IgE may contribute to the pathophysiology of airway dysfunction in children who develop this class of specific antibody.

Keywords: airway function; animal model; asthma; IgE; respiratory syncytial virus

Respiratory syncytial virus (RSV) is the most common cause of acute bronchiolitis that occurs during early life and is associated with subsequent development of persistent wheezing and asthma (1–4). Although the nature of this relationship remains to be fully defined, clinical studies have shown that children with severe RSV bronchiolitis requiring hospitalization are at high risk for developing childhood asthma (5–8). Whether RSV infection of the lower respiratory tract by itself can lead to long-term immunologic and physiologic airway dysfunction (the causal hypothesis) or whether such alterations result from a preexisting condition, possibly determined by a predisposing genetic susceptibility that is evoked by RSV infection (the association hypothesis), remains a matter of intense investigation and debate (9–13). Nonetheless, identifying host response factors that may contribute to the pathogenesis of post-RSV wheezing and asthma is critical for establishing a functional association between RSV-induced bronchiolitis and the subsequent development of persistent wheezing and asthma. Among these factors, RSV-specific IgE (RSV-IgE) antibody responses are of particular interest given the involvement of specific IgE antibodies in the pathophysiology of asthma (14).

RSV-IgE antibodies were first reported in 1981 by Welliver and colleagues who detected increased levels in nasopharyngeal secretions collected from children with documented RSV infection (15). RSV-IgE antibody responses appeared mostly in children who developed wheezing and were associated with increased levels of histamine in nasopharyngeal secretions and correlated with the degree of hypoxemia. Although some studies failed to detect RSV-IgE antibodies in nasal washes or sera from children with wheezing (16, 17), several reports have confirmed the presence of RSV-IgE antibodies in nasopharyngeal washes and sera collected from children with more severe illness caused by RSV infection (18–21).

Based on these clinical observations, we hypothesized that RSV-IgE antibodies might play a role in the development of exaggerated airway responsiveness during subsequent RSV infection. To test this hypothesis, we first defined the kinetics of the development of RSV-IgE antibody responses in a mouse model of RSV-induced airway inflammation and airway hyperresponsiveness (AHR). We then examined the effects of these antibodies *in vitro*, on mast cell activation and serotonin release and *in vivo* on RSV-induced airway inflammation and hyperresponsiveness. Some of the results of these studies have been previously reported in the form of an abstract (22).

METHODS

Additional detail on the methods is provided in an online supplement.

Animals

BALB/c mice (8–10 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). *FcεRI*^{−/−} mice, backcrossed on a BALB/c background, were generated as previously described (23). They were kindly provided by Dr. J. P. Kinet (Harvard Medical School, Boston, MA) and were bred under pathogen-free conditions in the animal facility at National Jewish Medical and Research Center (Denver, CO). The animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee.

Virus and Animal Inoculation

Human RSV, strain A2 (catalog #VR-1302), was obtained from American Type Culture Collection (Manassas, VA). Stocks of virus were purified by ultracentrifugation on sucrose density gradients. Animals were inoculated under light anesthesia (Avertin 2.5%, 0.010 ml/g body weight) with 10⁶ plaque-forming units of purified virus. Control animals received sham inoculum, consisting of virus-free preparation, or ultraviolet light (UV)-inactivated RSV (at a dose equivalent to 10⁶ plaque-forming units).

Analysis of mRNA Expression

Lung tissue mRNA expression was analyzed by the reverse transcription-polymerase chain reaction method using specific oligonucleotide primers (Table 1). Lung infection was confirmed by reverse transcription-polymerase chain reaction detection of RSV nucleoprotein gene (24). Polymerase chain reaction products were resolved by electrophoresis on ethidium bromide-stained agarose gels. The relative mRNA abundance for each target was determined by densitometric measurement of target to β-actin mRNA ratio using National Institutes of Health

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TABLE 1. OLIGONUCLEOTIDE PRIMERS

Target	GenBank	Sequence of Primer	Size of Fragment (bp)
β -actin	M12481	FP: 5'-TGTTACCAACTGGGACGACA-3' RP: 5'-AAGGAAGGCTGGAAAAGAGC-3'	573
IgE _{heavy} chain	Z27397	FP: 5'-CCTGGAAGCGAGAAGAATG-3' RP: 5'-CCAGCCACTGCACAGAGATA-3'	347
Fc ϵ RI α	J05018	FP: 5'-CTGGTGCAGTTAGCACCTGA-3' RP: 5'-TCTGAAGGAGCAGCCAATCT-3'	379
Fc ϵ RI β	J05019	FP: 5'-GCTCTCCCAATCCACAAGA-3' RP: 5'-CCGTCGTCTTCGGTTACATT-3'	452
Fc ϵ RI γ	J05020	FP: 5'-TGATCTCAGCCGTGATCTTG-3' RP: 5'-TTTCAGGTCTCTGGCAGCTT-3'	537
CD23	M34163	FP: 5'-GAGCCCTGTGGGTATAGCA-3' RP: 5'-AAAGCCTTCGGTCACAGAGA-3'	328
IL-4	M25892	FP: 5'-TCAACCCCGAGCTAGTTGTC-3' RP: 5'-TGGACTCATTGATGGTGCAG-3'	351
IL-13	NM_008355	FP: 5'-CAGCTCCCTGGTTCTCTCAC-3' RP: 5'-AGGCCATGCAATATCCTCTG-3'	309
CD40L	X65453	FP: 5'-TACTCAAGCGGCAATACC-3' RP: 5'-GAATCAGTGGGGTGTCTGTT-3'	419

Definition of abbreviation: IL = interleukin.

Scion Image software (version 1.62, developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

Detection of RSV-specific Antibodies

RSV-specific antibodies were detected by a direct ELISA in sera collected on Days 7, 14, 21, and 28 after RSV inoculation.

Generation of RSV-IgE and IgG2a/2b Antibodies

To generate mouse sera with high titers of RSV-IgE or IgG2a/2b (RSV-IgG2), mice were repeatedly sensitized by injection of 10 μ g of detergent-disrupted RSV antigens premixed with either alum adjuvant (AlumImuject; Pierce, Rockford, IL) or Freund's adjuvant (Sigma Chemicals Co., St. Louis, MO), respectively. For passive sensitization, RSV-IgE-enriched sera were depleted of total IgG (or IgE) antibodies by incubation with goat anti-mouse IgG (or IgE) immobilized on tissue culture plates. Complete depletion was confirmed by ELISA measurements. Normal mouse serum was similarly treated and used as control.

Mast Cell Degranulation

Bone marrow-derived mast cells (BMMCs) were generated by culture and used in a degranulation assay as previously described (25).

Airway Function, Bronchoalveolar Lavage, and Interferon- γ Levels

Airway function was assessed in nonanesthetized unrestrained animals by measuring changes in Penh (enhanced pause) values, using whole body plethysmography (26) and in anesthetized mechanically ventilated animals by measuring changes in lung resistance (27) in response to increasing doses of inhaled methacholine (MCh), as previously described in detail. Bronchoalveolar lavage (BAL) fluids were recovered for measurements of interferon- γ (IFN- γ) levels by ELISA (BD Pharmingen, San Diego, CA).

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical significance at a *p* value of less than 0.05 was determined by analysis of variance using Statview 4.5 statistical analysis software package (Abacus Concepts Inc., Berkeley, CA). The Bonferroni procedure was used to correct for multiple comparisons of the means.

RESULTS

RSV Infection Results in the Development of an RSV-IgE Antibody Response

The results of reverse transcription-polymerase chain reaction analysis revealed that RSV infection induced both interleukin

(IL)-4 and IL-13 mRNA expression in the lungs of these animals and upregulated the mRNA levels of CD40L, all considered to be critical signals required for IgE synthesis (Figures 1A and 1B). The data also show evidence for local IgE mRNA induction in the lungs of RSV-infected BALB/c mice (Figures 1C and 1D). In addition, both the high (Fc ϵ RI) and the low-affinity (CD23) receptors for IgE were upregulated at the mRNA level in RSV-infected mouse lungs.

To document further the development of RSV-IgE antibody responses in BALB/c mice, we established a direct ELISA method designed to detect circulating, free (unbound) RSV-specific antibodies in the sera collected from animals at 7, 14, 21, and 28 days after RSV infection. The results further confirmed the development of an RSV-IgE antibody response peaking at 21 days after inoculation in the sera of infected animals (Figure 2A); at these time points, IgG responses were also detected (Figure 2B). Incubation of samples from the same animal sera with ELISA plates coated with total HEp-2 cell protein extracts did not result in a significant binding of IgE or IgG antibodies (Figures 2C and 2D).

RSV-IgE Antibodies Trigger Mast Cell Degranulation in an IgE-dependent and Antigen-specific Manner

Mast cells are a potent source of mediators of bronchoconstriction (28). To establish a role for RSV-IgE antibodies in mast cell mediator release, we used an *in vitro* BMMC serotonin release assay (25).

To determine whether naturally occurring RSV-IgE antibodies can sensitize mast cells, BMMCs were sensitized with sera collected from RSV-infected mice and labeled with ^{3} H-serotonin. Serotonin release was measured in the cultures 30 minutes after addition of culture medium alone (spontaneous release) or either live or UV-inactivated RSV, diluted with culture medium to obtain a multiplicity of infection of three as shown in preliminary experiments to be an optimal dose for inducing maximal serotonin release. The results clearly demonstrate that RSV-IgE, generated under natural infection, can sensitize mast cells to degranulate after stimulation with RSV (Figure 3A). The release of serotonin from IgE-sensitized BMMCs was identical whether the cells were stimulated with UV-inactivated or live RSV, indicating that infection of these cells was not required for the response induced by RSV antigens. Furthermore, RSV alone did not

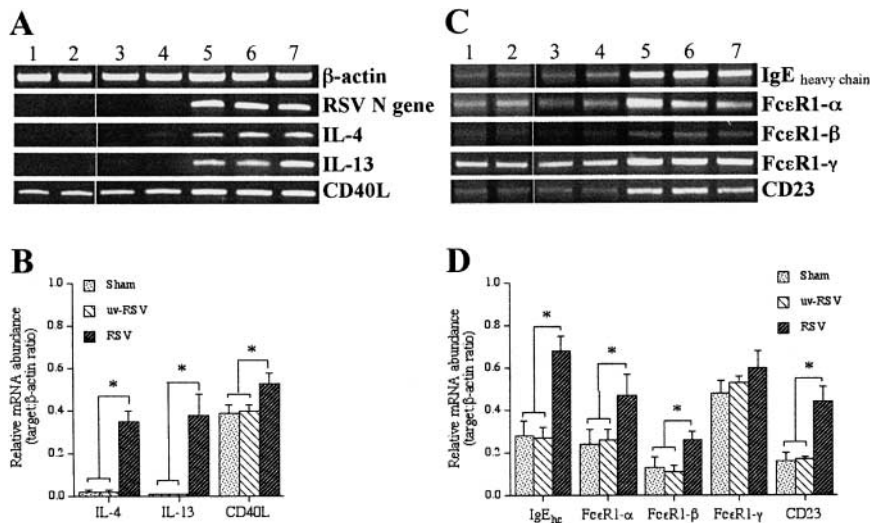


Figure 1. Respiratory syncytial virus (RSV)-mediated induction of mRNA expression for IgE and its receptors in the mouse lung. Representative agarose gels showing reverse transcription-polymerase chain reaction products (A and C) and relative mRNA abundance (B and D) for IgE, IgE receptors, interleukin (IL)-4, IL-13, and CD40L detected in the lungs of BALB/c mice inoculated with virus-free sham preparation (lanes 1 and 2), noninfectious, ultraviolet (UV)-inactivated RSV (lanes 3 and 4), or infectious RSV (lanes 5–7). RSV infection resulted in significant induction of mRNA for IgE, IgE receptors, and regulatory signals (IL-4, IL-13, CD40L) in the lungs of mice. Data are representative of two independent experiments with a total of four sham-inoculated animals, four UV-inactivated RSV (uv-RSV)-inoculated animals, and six live RSV-infected animals. *Significant difference between the groups ($p < 0.05$).

induce serotonin release from nonsensitized BMMCs (data not shown).

To demonstrate that naturally occurring RSV-IgE-mediated mast cell degranulation is IgE dependent, BMMCs were obtained from mice lacking the high-affinity receptor for IgE ($Fc\epsilon R1^{-/-}$) and were sensitized as described before. As shown in Figure 3A, RSV-infected animal sera failed to sensitize $Fc\epsilon R1^{-/-}$ BMMCs to degranulate after RSV stimulation, indicating that the mast cell response to RSV is IgE dependent.

To assess the antigen specificity of the IgE-mediated mast cell response, BMMCs were sensitized with IgG-depleted hyperimmune RSV-IgE-enriched serum, obtained by active sensitization of mice with RSV using alum adjuvant, or with a mouse monoclonal antiovalbumin IgE antibody, previously developed and characterized in this laboratory (29). The cells were stimulated with UV-inactivated RSV or ovalbumin followed by assessment of degranulation by measuring serotonin release. As shown in Figure 3B, RSV induced serotonin release from BMMCs, but only if these cells were sensitized with RSV-IgE but not with ovalbumin-specific IgE. Depletion of IgE from RSV-IgE serum abolished IgE-mediated mast cell degranulation and incubation of BMMCs with hyperimmune RSV-IgG2a/2b-enriched serum failed to mediate mast cell degranulation in response to RSV stimulation (data not shown). Furthermore, unlike ovalbumin, RSV did not stimulate serotonin release from BMMCs that were sensitized with ovalbumin-specific IgE (Figure 3B).

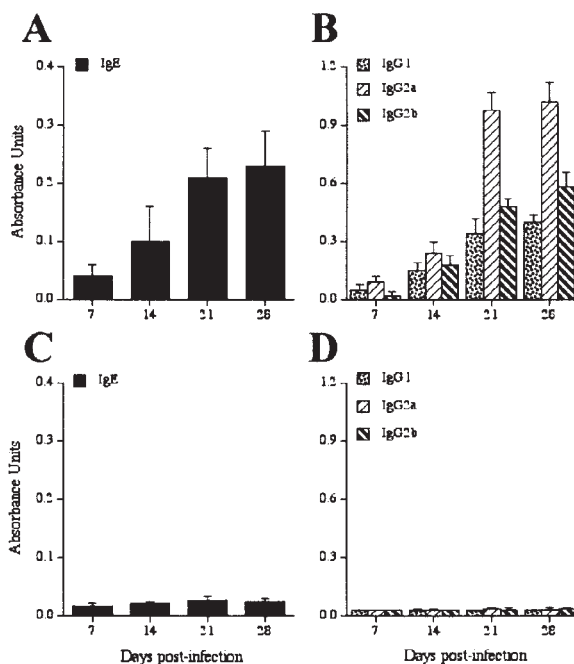


Figure 2. Kinetics of RSV-specific IgE (RSV-IgE) and IgG antibody responses in BALB/c mice. RSV-specific antibodies were measured in serum by direct ELISA. Paired samples from each individual mouse serum were incubated with ELISA plates coated with RSV antigens (A and B) to assess specific binding or with HEP-2 cell lysates (C and D) to assess nonspecific binding. Data are means $\pm$ SEM with $n = 8$ animals in each group. RSV infection resulted in the development of RSV-IgE (A) and IgG (B) antibody responses.

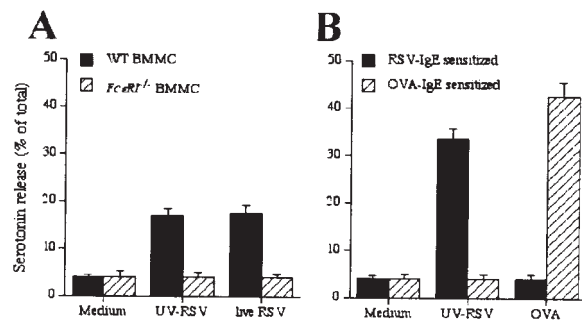


Figure 3. Effect of RSV-IgE on mast cell degranulation. (A) Naturally occurring RSV-IgE can sensitize mast cells from wild-type (WT) but not $Fc\epsilon R1^{-/-}$ mice to degranulate in the presence of RSV. Data are means $\pm$ SEM for $n = 6$ RSV-infected animal sera individually tested in triplicate cultures. (B) IgE-mediated mast cell degranulation is antigen specific. RSV-IgE mediates degranulation of mast cells only after stimulation with RSV but not with an irrelevant antigen such as ovalbumin (OVA). WT bone marrow-derived mast cells (BMMCs) were sensitized with hyperimmune RSV-IgE (IgG-depleted) serum or an OVA-specific monoclonal mouse IgE antibody. Data (mean $\pm$ SEM) are pooled from two independent experiments with quadruplicate cell cultures for each condition.

RSV-IgE Antibodies, not IgG2, Enhance RSV-induced AHR

To define the role of RSV-IgE in RSV-induced airway dysfunction, we passively sensitized BALB/c mice by intravenous administration of RSV-IgE or RSV-IgG2 sera 2 days after inoculation with live virus. In preliminary experiments, this time point showed maximal effects of passive sensitization on AHR. Control, sham-inoculated animals were administered normal BALB/c mouse serum. Airway responsiveness to inhaled MCh was measured by whole body plethysmography on Day 7 after infection. The results indicate that RSV-induced AHR was markedly enhanced by passive sensitization of mice with RSV-IgE antibodies (Figure 4A). If RSV-IgE was depleted from the serum, there was no enhancement of AHR. In contrast, mice administered RSV-IgG2 developed significantly lower AHR in response to RSV infection. As shown in Figure 4B, passive sensitization with RSV-IgE but not RSV-IgG2 resulted in markedly reduced numbers of lymphocytes recovered in the BAL fluids from RSV-infected animals.

To determine whether infectious RSV was required for the IgE-mediated enhancement of AHR, BALB/c mice were inoculated with UV-inactivated RSV on Day 0 or Day 3 and were passively sensitized on Day 2 with RSV-IgE, followed by assessment of airway responsiveness to MCh on Day 7. Under each condition, no significant increase beyond normal airway responsiveness could be observed, demonstrating that active RSV lung infection was required for the enhanced response (Figure 4C). Similarly, no significant changes were seen between these groups in the profile of inflammatory cell populations recovered in the BAL fluids (Figure 4D). In addition, the normal airway responsiveness to MCh seen in sham-inoculated mice was not altered by passive sensitization of the mice with either RSV-IgE or RSV-IgG2 (data not shown), indicating that interaction of these antibodies with RSV *in vivo* during active lung infection is necessary to produce the exacerbated AHR.

To assess further the antigen specificity of IgE-mediated enhancement of RSV-induced AHR *in vivo*, mice were either actively sensitized twice to ovalbumin and infected with RSV 14 days after the second sensitization or passively sensitized 2 days

after RSV infection by administration of ovalbumin-specific mouse monoclonal IgE antibody. In parallel, a group of sham-inoculated mice received RSV-IgE on Day 2 followed by intranasal administration of ovalbumin (50 μ g in 25- μ l saline) on Day 3 after inoculation. Control groups consisted of sham-inoculated and RSV-infected mice that received normal serum as described previously here. AHR and airway inflammation were assessed on Day 7 after inoculation by measuring changes in lung resistance to inhaled MCh. The data demonstrate that RSV-induced AHR is not enhanced after passive sensitization with IgE antibodies that are specific to an irrelevant antigen such as ovalbumin (Figure 5A). Similarly, an irrelevant antigen (ovalbumin) did not induce AHR in mice sensitized with RSV-IgE. As shown in Figure 5B, the profile of inflammatory cells recovered in the BAL fluids of RSV-infected animals was not altered by active sensitization to ovalbumin or by passive sensitization with ovalbumin-specific monoclonal IgE antibody. Similarly, the profile of the BAL cell population in the sham-inoculated animals was not altered after sensitization with RSV-IgE.

The High-affinity IgE Receptor (Fc ϵ RI) Is Required for the Enhancement of RSV-induced AHR by RSV-IgE

The high-affinity IgE receptor, Fc ϵ RI, plays an essential role in mast cell activation and mediator release. To define the role of Fc ϵ RI *in vivo*, in the enhancement of RSV-induced AHR by RSV-IgE, we passively sensitized *Fc ϵ RI*^{-/-} mice, on Day 2 after inoculation, by intravenous administration of either normal serum or RSV-IgE. Wild-type (WT) mice were similarly treated and served as controls for passive sensitization. Airway function was assessed on Day 7 after RSV inoculation in anesthetized, mechanically ventilated animals by measuring changes in lung resistance to increasing doses of inhaled MCh. Compared with WT mice (Figure 6A), *Fc ϵ RI*^{-/-} mice developed a lower degree of AHR on Day 7 after RSV infection (Figure 6D). Nonetheless, the increases in AHR were significantly higher than in control animals, which received sham inoculation. However, after passive sensitization of *Fc ϵ RI*^{-/-} mice with RSV-IgE, no further increases in lung resistance were observed after RSV infection (Figure 6D),

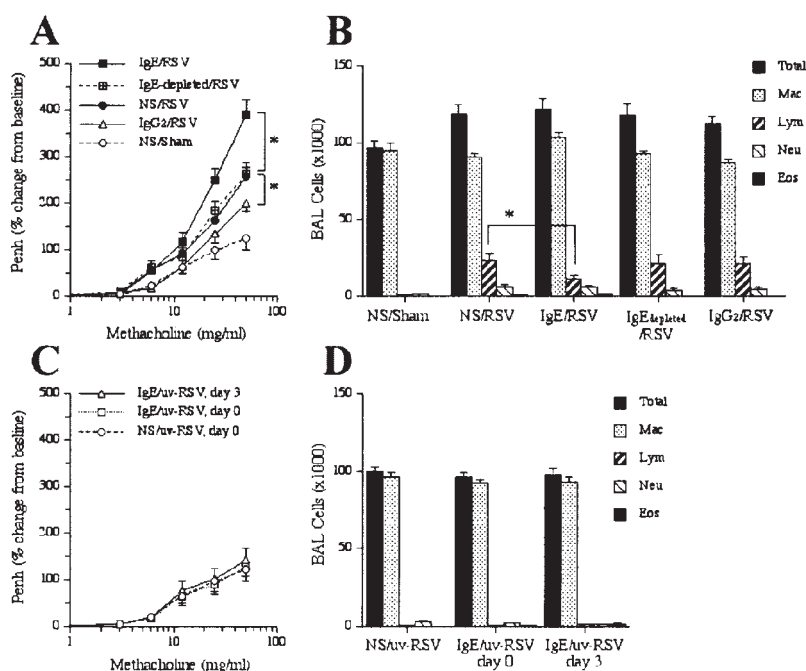


Figure 4. Effect of passive sensitization with RSV-IgE antibodies on RSV-induced airway hyperresponsiveness (AHR) (A and C) and airway inflammation (B and D). (A) Mice were inoculated on Day 0 with RSV and administered intravenously on Day 2 IgG-depleted RSV-IgE-enriched serum (IgE/RSV), IgE-depleted RSV-IgE-enriched serum (IgE depleted/RSV), RSV-specific IgG2 (IgG2/RSV), or normal serum (NS/RSV). Control mice (NS/Sham) were sham-inoculated and treated with normal serum. (B) Profile of inflammatory cells recovered in the bronchoalveolar lavage (BAL) fluids from the same groups of mice (as in A). (C) Mice were inoculated on experimental Day 0 with UV-inactivated RSV followed on Day 2 by an intravenous administration of either normal serum (NS/uv-RSV, Day 0) or RSV-IgE (IgE/uv-RSV, Day 0); other mice were inoculated on Day 3 with UV-inactivated RSV after administration on Day 2 of RSV-IgE (IgE/uv-RSV, Day 3). (D) Profile of inflammatory cells recovered in the BAL fluids from the same groups of mice (as in C). Data are means $\pm$ SEM with $n = 8$ animals in each group. *Significant differences between the groups, $p < 0.05$. Eos = eosinophil; Lym = lymphocyte; Mac = macrophage; Neu = neutrophil.

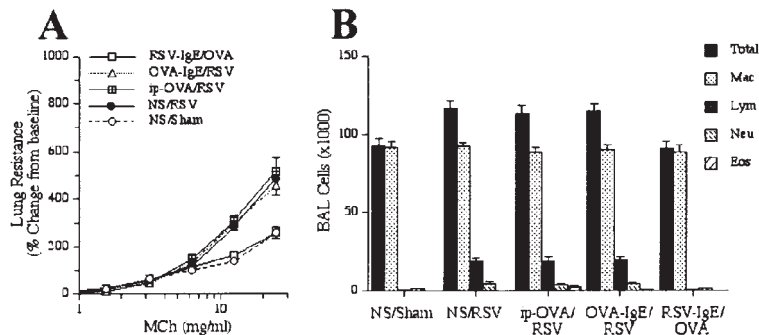


Figure 5. Antigen specificity of IgE-mediated enhancement of RSV-induced AHR. (A) Airway responsiveness to inhaled methacholine (MCh). Groups of mice were administered RSV-IgE and intranasally challenged with OVA (RSV-IgE/OVA, $n = 6$), administered monoclonal IgE antibody and infected with RSV (OVA-IgE/RSV, $n = 6$), actively sensitized to ovalbumin and infected with RSV (intraperitoneal-OVA/RSV, $n = 6$), or administered normal serum and infected with RSV (NS/RSV, $n = 6$) or sham inoculated (NS/Sham, $n = 6$). (B) Profile of inflammatory cells recovered in the BAL fluids from the same groups of mice.

demonstrating that the high-affinity IgE receptor is required for the enhanced response. In contrast, passive sensitization of WT mice with RSV-IgE significantly enhanced RSV-induced AHR (Figure 6A), further confirming the airway physiology data obtained by whole body plethysmography (shown in Figure 4A). Analysis of inflammatory cells recovered in the BAL fluids revealed significantly reduced numbers of lymphocytes after passive sensitization with RSV-IgE in WT (Figure 6B), but not in *FcεRI*^{-/-} mice (Figure 6E).

To determine the effects of RSV-IgE antibodies on viral clearance, lung viral titers were measured on Days 4 and 7 after infection for both strains of mice. The results showed no significant differences between the two strains of mice in the amounts of replicating virus recovered from the lungs (Figures 6C and 6D). Furthermore, in both strains of mice, the lung viral titers were not affected by passive sensitization with RSV-IgE.

Passive Sensitization with RSV-IgE Antibodies Decreases RSV-induced IFN- γ Levels

IFN- γ may play a significant role in the pathogenesis of RSV bronchiolitis (30). To define the effects of RSV-IgE antibodies on the IFN- γ response to RSV lung infection, we measured the levels of this cytokine in the BAL fluids recovered from both

WT and *FcεRI*^{-/-} mice after RSV infection and passive sensitization with RSV-IgE antibodies. RSV infection led to a marked increase in IFN- γ levels in both strains of mice, with WT mice showing significantly higher levels than *FcεRI*^{-/-} mice (Figure 7). However, passive sensitization with RSV-IgE resulted in significantly lower IFN- γ production in the lungs of WT mice (Figure 7A) but not in the *FcεRI*^{-/-} mice (Figure 7B).

DISCUSSION

The nature of the clinical association that exists between RSV bronchiolitis and subsequent development of wheezing and asthma in young children remains controversial. However, genetic susceptibility and host response factors are likely to play important roles in the pathogenesis of postbronchiolitis wheezing and asthma. In this study, we investigated the role of RSV-IgE antibodies in the pathophysiology of RSV-mediated airway dysfunction. Consistent with clinical reports from human studies, the results of this animal study confirmed that the development of RSV-IgE antibodies is a component of the host response to RSV infection. The peak levels of circulating, free (unbound) RSV-IgE antibodies were detected in serum at 3 weeks after infection. Similar findings have been reported earlier by other studies conducted in different animal species including calves (31) and guinea pigs (32).

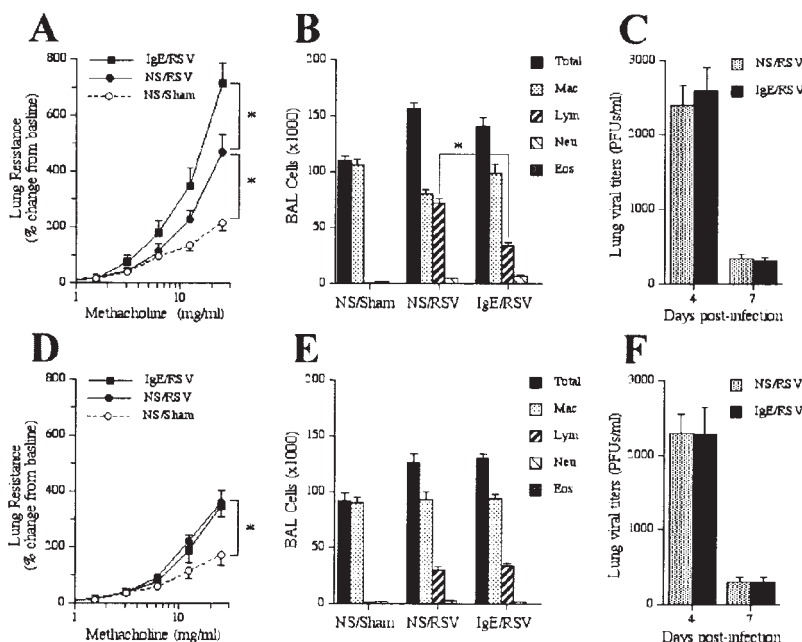


Figure 6. Requirement for the high-affinity IgE receptor (*FcεRI*). WT BALB/c mice (A–C) and *FcεRI*^{-/-} mice (D–F) were inoculated on experimental Day 0 with RSV and administered on Day 2 RSV-IgE-enriched serum (IgE/RSV) or normal mouse serum (NS/RSV). Control mice were sham-inoculated and treated with normal mouse serum (NS/Sham). Airway function was assessed on Day 7 in anesthetized, mechanically ventilated animals by measuring changes in lung resistance to inhaled MCh (A and D), followed by total and differential BAL cell counts for the same groups animals (B and E). Lung viral titers were determined on separate animal groups on Days 4 and 7 after infection by culture plaque assays (C and F). Data (mean $\pm$ SEM) are presented for $n = 8$ animals in each group. *Significant difference between the groups, $p < 0.05$.

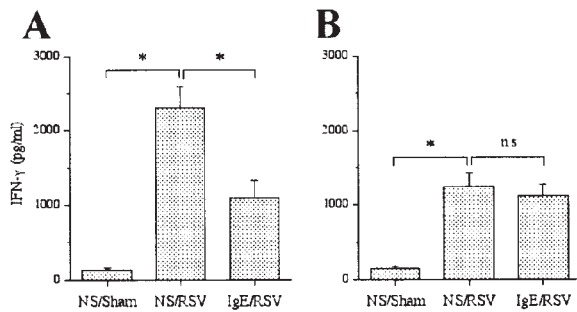


Figure 7. Effect of RSV-IgE on IFN- γ production *in vivo*. The treatment groups are the same as in Figure 6. Interferon- γ (IFN- γ) levels were measured by ELISA in the BAL fluids recovered on Day 7 after infection from the lungs of BALB/c mice (A) and *FcεRI*^{-/-} mice (B). *Significant difference between the groups, $p < 0.05$; n.s. = not significant.

The development of virus-specific IgE antibodies is not unique to RSV infection. Numerous human studies have also shown that specific IgE antibodies can be produced in response to infection by other pathogens, including a variety of viruses (33–39) and atypical bacteria (40, 41). However, the role of pathogen-specific IgE antibodies has not been determined in these studies, although when studied, in most cases, the responses were associated with a poorer prognosis.

The induction of an IgE antibody response is T-helper cell dependent and requires a primary signal delivered by a Th2 cytokine IL-4 (or IL-13) and a secondary signal delivered by the interaction between CD40, expressed on B cells, and its natural ligand (CD40L) expressed on activated T cells (42, 43). Once initiated, IL-4-driven IgE synthesis can either be enhanced by Th2 cytokines (IL-5, IL-6, and IL-9) or inhibited by Th1 cytokines (IFN- γ) or antiinflammatory cytokines such as IL-10 and transforming growth factor- β (44). Thus, Th2 cells, and possibly basophils and mast cells, which may constitute a considerable source of IL-4 and IL-13 (45), are crucial for the control of IgE production. Accordingly, the initial induction of RSV-IgE antibody production is likely to be dependent on a Th2 response that develops during RSV infection, albeit discrete, and is most likely compartmentalized to the lung tissue.

Because some studies have failed to detect Th2 cytokines in clinical samples obtained from children with RSV infection, the issue as to whether natural RSV infection can elicit a Th2 response remains a matter of debate. Nonetheless, the ability of RSV to induce Th2 cytokine production is well documented (46–48), and there are consistent data from clinical studies that described an otherwise low level but significant Th2 response that is associated with RSV bronchiolitis (49–54). Some animal studies suggest that the extent of these responses could be limited because of the predominant IFN- γ production induced during acute RSV infection (55). In this study, the results of reverse transcription-polymerase chain reaction analysis of mRNA expression revealed that both critical signals required for IgE synthesis, including CD40L and the Th2 cytokines IL-4 and IL-13, were upregulated in the lungs of mice during RSV infection. Furthermore, the expression levels for both the low-affinity (CD23) and the high-affinity (FcεRI) IgE receptors were also increased in the lungs of RSV-infected mice.

Based on the similarities between postbronchiolitic wheezing and asthma and the well known involvement of IgE in the pathogenesis of asthma (14), we hypothesized that RSV-IgE antibodies could play a significant role in the pathophysiology of RSV-mediated airway dysfunction. Our *in vitro* studies demonstrated

that cross-linking of RSV-IgE antibodies on the surface of sensitized mast cells triggers a degranulation response and the release of serotonin. We therefore postulated that if present *in vivo*, RSV-IgE antibodies might trigger similar responses that could result in the development or enhancement of a bronchoconstrictive airway response after RSV infection. As expected, mice that were passively sensitized with RSV-IgE antibodies developed further increases in airway responsiveness to inhaled MCh after RSV infection as compared with RSV-infected mice that were treated with normal serum or RSV-IgE-depleted serum. Surprisingly, noninfectious (UV-inactivated) virus failed to induce AHR after passive sensitization of mice with RSV-IgE antibodies (Figure 4). This is in contrast to our *in vitro* data where both infectious and noninfectious RSV induced equal amounts of serotonin release from sensitized mast cells. However, the differences observed *in vivo* may be related to the requirement of infectious virus to upregulate IgE receptor expression in the lungs. On the other hand, passive sensitization of *FcεRI*^{-/-} mice with RSV-IgE antibodies failed to enhance RSV-mediated AHR, further identifying a critical role for the high-affinity IgE receptor in the *in vivo*-mediated effects on AHR.

In mice, acute RSV lung infection typically results in the development of a predominantly mononuclear cell inflammation that can be sampled in the BAL fluid. In this study, the number of lymphocytes recovered in the BAL fluid was significantly lower in RSV-infected BALB/c mice administered RSV-IgE antibodies. This decrease in the cellular response was associated with lower levels of IFN- γ in the recovered BAL fluids. However, the decreases were not reflected in altered lung viral titers, which were not affected by passive sensitization with RSV-IgE antibodies. In contrast, in RSV-infected *FcεRI*^{-/-} mice, neither the numbers of recovered lymphocytes nor the levels of IFN- γ were decreased in the BAL after passive sensitization with RSV-IgE antibodies.

Deficient IFN- γ production has been associated with RSV infection and correlated with severity of disease in human studies (18, 56–61). In animal studies, deficient IFN- γ production by RSV-specific CD8⁺ T cells has been related to the inability of these cells to mount appropriate effector and memory cytolytic responses (30), although viral clearance can be achieved in the absence of CD8⁺ T cells (62). A more recent study emphasized the importance of IFN signaling pathways in the development of Th1-protective immunity against RSV infection (63). The authors described a strong Th2-biased, RSV-induced lung histopathology in mice lacking either the intracellular STAT1 signaling molecule or both of IFN- γ and IFN- α/β receptors. Because there are multiple cellular sources of IFN- γ production in the lungs, including natural killer cells (64), and both $\alpha\beta$ and $\gamma\delta$ TCR⁺ T cells (56) and a variety of cells other than mast cells and basophils, including monocytes (65), eosinophils (66), dendritic cells (67, 68), and airway epithelial cells (69) can also express the high-affinity receptor for IgE, further studies are required to elucidate fully the mechanisms whereby RSV-IgE antibodies mediate lower IFN- γ production in the lungs and enhanced airway dysfunction after acute RSV infection.

In summary, the results of this study document the development of RSV-IgE responses in RSV-infected BALB/c mice and establish a role for these antibodies and the high-affinity IgE receptor in the development of exaggerated AHR after active RSV lung infection. Because recurrent RSV infection is common in children (70), the described interactions might contribute to the development of airway dysfunction in children who have developed RSV-IgE antibodies.

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The role of Virus-Specific Immunoglobulin E in Airway Hyperresponsiveness

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“Online Data Supplement”

MATERIALS AND METHODS

Virus preparation. The strain A2 of human RSV (Cat. # VR-1302) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Stocks of RSV were prepared under endotoxin-free conditions and were assessed to be free of endotoxin contamination using a *Limulus* Amoebocyte Lysate (LAL)-based Pyrogen® Plus Gel Clot assay with a sensitivity of 0.06 endotoxin units/ml (BioWhittaker Inc., Cat # N283-06, Walkersville, MD, USA). The virus was propagated in monolayers of HEp-2 cells (ATCC, Cat. # CCL-23) grown in Eagle's minimum essential medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS). At maximum cytopathic effect, the cells were harvested and disrupted by sonication in the same culture medium. The suspension was clarified by centrifugation at 10,000 x *g* for 15 minutes at 10°C and the resulting supernatant was layered on top of a sucrose gradient (30% in 50 mM Tris-buffered normal saline solution containing 1 mM ethylenediaminetetraacetate [EDTA], pH 7.5) and further centrifuged at 100,000 x *g* for 2 h at 10°C. The pellet containing purified virus was resuspended in 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 15% sucrose and stored in aliquots at -70°C. Viral titers were determined by standard plaque assay combined with immunostaining for RSV using a biotinylated goat anti-human RSV antibody (Accurate Chemical & Scientific Corp., Westbury, NY, USA) and an Avidin-Biotin Peroxidase detection system (DAKO Corp., Carpinteria, CA, USA). Uninfected HEp-2 cell cultures were similarly processed to obtain a control, virus-free sham preparation.

Mice were inoculated under light anesthesia (Avertin 2.5%, 0.010 ml/g body weight) by intranasal instillation of 25 µl endotoxin-free PBS containing 10⁶ plaque-forming units (PFU) of

purified RSV. Control mice were inoculated under the same conditions with the sham preparation or ultra-violet light (UV)-inactivated RSV (at a dose equivalent to 10^6 PFUs). Full inactivation of RSV, as assessed by plaque assay, was achieved by exposing the virus in a sterile glass vial to a UV light source (302 nm Chromato-Vue Lamp UVM-57, San Gabriel, California) for 16 h at 4°C.

Analysis of messenger RNA (mRNA) expression. Lung tissue mRNA expression was analyzed by the reverse transcription and polymerase chain reaction (RT-PCR) method. Total RNA was extracted from each individual mouse lung using Trizol Reagent (Invitrogen Corp.) and cleaned up with RNeasy mini columns (QIAGEN Inc., Valencia, CA, USA). Ten micrograms of extracted RNA were reverse-transcribed into cDNA using M-MLV reverse transcriptase and oligo (dT)₁₂₋₁₈ primers (Invitrogen Corp.), followed by 30 cycles of PCR amplification in iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) using specific oligonucleotide primers and *Taq* DNA polymerase (Invitrogen Corp.). The PCR products were resolved by electrophoresis on ethidium bromide-stained agarose gels and visualized by ultraviolet light illumination. Images of the gels were captured with a digital camera and transferred to a G4 Macintosh computer for densitometric measurements of the signal intensity for each band. The relative mRNA abundance for each target was determined by measuring the ratio of target to β -actin mRNA signals using NIH *Scion Image* software (version 1.62, developed at the U. S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

Detection of RSV-specific antibodies. RSV-specific antibodies were detected by a direct enzyme-linked immunosorbent assay (ELISA), in sera collected on days 7, 14, 21 and 28 following RSV inoculation. Unless otherwise specified, all incubations were carried out at room temperature. The ELISA plates were coated overnight at 4°C with detergent-disrupted RSV

antigens or HEp-2 cell lysate (10 µg/well in 0.1 M carbonate/bicarbonate coating buffer, pH 9.6) and washed four times with ELISA wash buffer (0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween-20). After blocking for 2 h with assay buffer (PBS containing 1% bovine serum albumin (BSA)) to prevent nonspecific binding, the sera were diluted 1:100 in assay buffer and applied to the plates for a 2-h incubation followed by four washes as described above. RSV-specific mouse IgG1, IgG2a and IgG2b antibodies bound on the plate were detected with subclass-specific HRP-conjugated goat anti-mouse antibodies (SouthernBiotech, Birmingham, AL, USA). RSV-specific IgE antibodies were detected with a biotinylated rat monoclonal anti-mouse IgE antibody and a streptavidin-horseradish peroxidase conjugate (BD PharMingen, San Diego, CA, USA). After six washes, a soluble peroxidase substrate (BD PharMingen) was added for development and the reaction was stopped by addition of 2N H₂SO₄ solution, followed by reading of optical density (O.D.) at 450 nm. Sera from naïve and sham-inoculated mice were used as negative controls. An O.D. reading of 2 standard deviations above the average control values was retained as positive.

Generation of RSV-specific IgE and IgG2a/2b antibodies. RSV-specific mouse IgE antibodies were produced by actively sensitizing mice by intraperitoneal injection of 10 µg of detergent-disrupted RSV antigens pre-mixed with 2.25 mg alum (AlumImuject; Pierce, Rockford, IL, USA) in a total volume of 100 µl on days 1, 14 and 28. Sera were collected on day 32, pooled together and stored in aliquots at –80°C until needed for experiments. This sensitization protocol resulted in a predominant production of circulating IgG1 and IgE antibodies. As assessed by ELISA, the titers exceeded 1/1,000 for RSV-specific IgE.

For passive sensitization, RSV-specific IgE enriched sera were depleted of mouse IgG antibodies by three consecutive incubations (30 minutes each) with a biotinylated goat anti-

mouse IgG (SouthernBiotech), pre-adsorbed on streptavidin-coated plates. This antibody binds all mouse IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) but does not bind mouse IgE. Similarly, IgE antibodies were selectively depleted by incubation of immune serum with a biotinylated rat anti-mouse IgE (BD PharMingen) pre-adsorbed on streptavidin-coated plates. Depletion of IgG or IgE antibodies, from the respective sera, was confirmed by ELISA assays. Normal mouse serum was similarly treated and used as control.

RSV-specific IgG2a/2b enriched sera were prepared from mice actively sensitized by intraperitoneal injection of 100 μ l of an emulsion containing 10 μ g of detergent-disrupted RSV antigens in 50 μ l PBS pre-mixed with 50 μ l of complete Freund's adjuvant (Sigma Chemicals Co., St. Louis, Missouri) on days 1 and 14, followed by injections on day 28 of RSV antigens emulsified in incomplete Freund's adjuvant. Sera were collected on day 32 and stored at -80°C . Under these conditions, the mice produced high titers of RSV-specific IgG2a and IgG2b that exceeded 1/15,000 for each antibody subclass as determined by ELISA; no RSV-specific IgE or IgG1 was detected in these samples.

Active sensitization to ovalbumin. Mice were actively sensitized by two intraperitoneal injections, 2 weeks of apart, of 20 μ g of ovalbumin (Grade V, Sigma Chemicals Co., St. Louis, MO, USA), premixed with 2.25 mg aluminum hydroxide (AlumInject: Pierce, Rockford, IL, USA) in 100 μ l of endotoxin-free saline.

Mast cell degranulation assay. Fully differentiated bone marrow-derived mast cells (BMMCs) were resuspended at a density of 10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% FBS, 50 μ M of 2-mercapto-ethanol, 100 U/ml of penicillin, 100 μ g/ml of streptomycin, 0.5 μ g/ml amphotericin B and 10 μ g/ml of polymyxin B (Invitrogen

Corp.). The cells were sensitized by overnight incubation at 37°C with RSV-specific IgE (IgG-depleted) or IgG2a/2b enriched sera (diluted 1:100 in culture medium), or an ovalbumin-specific mouse monoclonal IgE antibody (0.5 µg/ml) and concomitantly labeled with 1 µCi of ³H-serotonin (5-hydroxy [G-³H] tryptamine creatinine sulphate, specific activity: 8.2 Ci/mmol) (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Controls consisted of non-sensitized BMMCs that were incubated under the same conditions with normal (naïve) mouse serum or with RSV-specific IgE enriched serum that was depleted of IgE, and similarly labeled with ³H-serotonin. For RSV-induced serotonin release, sensitized as well as non-sensitized BMMCs were washed twice and placed in 5-ml cell culture tubes containing culture medium. The cells were stimulated with either live, infectious RSV or UV-inactivated RSV at a dose of 3 multiplicities of infection (MOI) for 30 minutes. For spontaneous serotonin release, the cells were incubated with culture medium alone. Serotonin release was assessed by counting radioactivity in the culture supernatants, collected after centrifugation of cell suspensions at 1,200 rpm for 5 minutes, and was expressed as percent of total serotonin uptake determined prior to stimulation by counting total radioactivity for each sample after cell lysis with 1% Triton-X100.