

Allergic sensitization increases airway reactivity in guinea pigs with respiratory syncytial virus bronchiolitis

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Background: Respiratory syncytial virus (RSV) causes acute bronchiolitis in children and has been implicated in the pathogenesis of recurrent wheezing and asthma. However, few children exposed to RSV experience acute bronchiolitis or its sequelae, suggesting a subgroup of susceptible children. An allergic diathesis may predispose children to subsequent airway disease.

Objective: This study was carried out to determine whether a preexisting allergic state, induced by repeated inhalational exposures to ovalbumin, potentiates nonspecific airway responsiveness to acetylcholine and increases airway inflammation during acute RSV bronchiolitis in guinea pigs.

Methods: Forty guinea pigs were randomized into four groups: nonsensitized, noninfected (ovalbumin-, RSV-); sensitized, noninfected (ovalbumin+, RSV-); nonsensitized, infected (ovalbumin-, RSV+); sensitized, infected (ovalbumin+, RSV+). Depending on grouping, animals were exposed to either repeated aerosols of ovalbumin or saline solution and were subsequently inoculated with either human RSV or uninfected culture medium. Six days after inoculation, animals underwent acetylcholine challenge, and lung specimens were prepared for histologic scoring of airway inflammation.

Results: Maximal increases in pulmonary resistance (centimeters of water per milliliter per second) to acetylcholine were greater for RSV alone (12.4 ± 3.9) and ovalbumin alone (13.7 ± 3.9) compared with controls (4.3 ± 1.1), but significantly greater increases occurred in ovalbumin+, RSV+ animals (34.0 ± 11.0). These ovalbumin+, RSV+ animals demonstrated the combined histologic changes noted with RSV alone and ovalbumin alone including airway epithelial necrosis, mononuclear and granulocyte infiltrates, airway wall edema, hyperplasia of bronchus-associated lymphoid tissue, and goblet cell metaplasia.

Conclusion: Prior allergic sensitization potentiates the physiologic and structural changes induced by acute RSV bronchiolitis. These results suggest that an allergic diathesis may increase the severity of RSV infections in children. (*J Allergy Clin Immunol* 1997;100:492-8.)

Key words: Acute bronchiolitis, allergic sensitization, animal model

Respiratory syncytial virus (RSV) is the most common cause of acute bronchiolitis, a lower respiratory tract infection that results in the hospitalization of approximately 1% of children under the age of 2 years. Several studies have reported a high incidence of recurrent wheezing and asthma in infants hospitalized for treatment of acute bronchiolitis, which is frequently associated with allergy.¹⁻³ However, the mechanisms of acute bronchiolitis and its sequelae are poorly understood, given that most children show serologic evidence of RSV exposure during infancy. In particular, whether acute bronchiolitis represents an exaggerated response to RSV infection within a subgroup of allergic children remains unclear.⁴

A recent study in guinea pigs has demonstrated that acute parainfluenza type 3 virus infection potentiates increases in tracheal responsiveness to bronchoconstrictor agonists in vitro in animals that were presensitized to ovalbumin.⁵ These observations suggest that a preexisting allergic state may result in an exaggerated response to viral respiratory tract infection, as has been reported in patients with asthma.⁶ However, it remains uncertain whether acute viral infection produces similar effects on airway responsiveness in sensitized animals in vivo or whether any changes in airway responsiveness are accompanied by changes in lung histopathology. We have previously shown that juvenile guinea pigs infected with human RSV develop histologic features of acute bronchiolitis in association with increased airway reactivity to inhaled acetylcholine.^{7,8} We have also shown that guinea pigs repeatedly exposed to ovalbumin become sensitized and develop increased airway reactivity to inhaled acetylcholine and lung tissue eosinophilia.⁹ We therefore decided to investigate whether the effects of acute RSV infection on changes in airway physiology and lung histopathology in vivo would be more pronounced in guinea pigs that were sensitized to ovalbumin before viral infection.

METHODS

Animals

Juvenile, 8-week-old Cam Hartley guinea pigs (Charles River Laboratories, Montreal, Quebec, Canada) (mean weight $\pm$ SD: 484 ± 37 gm) were randomly assigned to four groups, each consisting of 10 animals. The groups were designated: (1)

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Abbreviations used

BALT:	Bronchus-associated lymphoid tissue
R _L :	Pulmonary resistance
RSV:	Respiratory syncytial virus

nonsensitized, non-RSV-infected (ovalbumin⁻, RSV⁻); (2) ovalbumin-sensitized, non-RSV-infected (ovalbumin⁺, RSV⁻); (3) nonsensitized, RSV-infected (ovalbumin⁻, RSV⁺), and (4) ovalbumin-sensitized, RSV-infected (ovalbumin⁺, RSV⁺). Animals were housed in rooms equipped with air filters in plastic cages containing corncob bedding, access to guinea pig chow, alfalfa hay cubes, and water with 12-hour alternating light-dark cycles. Animals were maintained in accordance with standards of the Canadian Council in Animal Care.

Protocol

Fig. 1 provides a schematic diagram of the time course of sensitization and viral inoculation.

Allergic sensitization

Animals were sensitized and repeatedly challenged with aerosolized ovalbumin as has been previously reported from this laboratory.⁹ Briefly, animals were sensitized to ovalbumin by aerosol administration of 1% ovalbumin and 4% heat-killed, *Bordetella pertussis* vaccine (Connaught Laboratories, Toronto, Ontario, Canada). Seven days after sensitization, animals received twice-weekly exposures to 0.5% ovalbumin aerosol for 4 to 5 weeks. Diphenhydramine (8 mg) was administered by intraperitoneal injection 1 hour before each exposure to avoid anaphylactic reactions. Exposure to RSV or uninfected cell culture supernatant (see below) occurred after the seventh ovalbumin challenge. The last ovalbumin challenge was given 2 days before the measurement of airway reactivity.

RSV inoculation

Animals were inoculated with 4000 plaque-forming units of human, Long strain, type A RSV as previously described.⁷ Animals were anesthetized with halothane, and 0.1 ml of virus was instilled onto the nasal mucosa. Control animals were similarly anesthetized but had 0.1 ml of sterile HEP-2 cell culture supernatant instilled intranasally. All physiologic measurements were performed 6 days after the inoculation procedure, which was chosen on the basis of our previous observations of maximal nonspecific airway responsiveness to acetylcholine challenge at this time in RSV-infected guinea pigs.⁸

Assessment of in vivo airway responsiveness

Six days after the inoculation procedure, animals were anesthetized with 0.3 ml of ketamine by intraperitoneal injection. After supplemental halothane anesthesia had been induced, a tracheostomy was performed, and the animals were placed in a pressure-sensitive plethysmograph, designed for the guinea pig, and ventilated with a Harvard animal ventilator (model 608; Harvard Apparatus, South Natick, Mass.), delivering a tidal volume of 3 ml at a frequency of 60 breaths/min. Animals were paralyzed with succinylcholine (0.5 mg/kg, given intramuscularly) immediately after attachment to the ventilator. Volume signals were obtained from a pressure-sensitive transducer (MP 45-2 $\pm$ 2 cm H₂O; Validyne, Northridge, Calif.), which mea-

sured changes in box pressure. The plethysmograph was calibrated with a 10 ml syringe, and a 3 ml volume change resulted in a 0.3 cm H₂O pressure change within the box. The pressure response was linear to a volume change of 20 ml. The volume signal was electronically differentiated to determine flow. Transpulmonary pressure was determined by using a differential pressure transducer (model 267BC; Hewlett Packard, Waltham, Mass.) by comparing pressure at the tracheal opening with esophageal pressure measured from a saline solution-filled PE-90 tube (Fisher Scientific, Nepean, Ontario, Canada) in the distal esophagus. The pressure transducer was calibrated by using a water-filled manometer. The response was linear over the range of -50 to +50 cm H₂O. The volume, flow, and pressure signals were recorded (General Scanning, model RS4-5P recorder), and pulmonary resistance (R_L) was calculated at 50% of tidal volume from these signals by using the method of von Neergaard and Wirz¹⁰ by a computer program developed in this laboratory.¹¹ After stable baseline R_L values were obtained, guinea pigs were given isotonic saline aerosol, followed by increasing doses of aerosolized acetylcholine, and the changes in R_L were measured.

Lung mechanics measurement

Acetylcholine chloride (Sigma Chemical, St. Louis, Mo.) was dissolved in normal saline solution to produce a stock solution of 50 mg/ml. Serial dilutions were made to produce solutions of 50, 15, 5, 1.5, and 0.5 mg/ml. An aerosol was generated by placing 5 ml of saline or acetylcholine solution in a Hudson nebulizer (Hudson Oxygen Therapy Sales Co., Temecula, Calif.) driven by compressed air at 7 L/min and connected by a T piece to the attachment tube for the tracheostomy. The ventilator was disconnected from the circuit during the administration of acetylcholine. Six 3 ml tidal breaths of nebulized acetylcholine were delivered for each concentration of acetylcholine. After the administration of each dose, the ventilator was immediately reconnected to the circuit. The computer analyzed the first 20 breaths after commencement of ventilation. Peak responses after acetylcholine challenges generally occurred within the first 10 breaths after reconnection to the ventilator. The average of the three highest R_L values at 50% tidal volume was taken as the response to each acetylcholine concentration. The next dose was delivered after R_L had returned to baseline.

Specimen preparation

After completion of the acetylcholine dose-response curve, the animal was removed from the ventilator and killed by exsanguination. The chest was opened under sterile conditions, and the lungs and heart were removed en bloc. The right main bronchus was identified and ligated, and the right lung was resected. The middle lobe was processed for viral culture in HEP-2 cell monolayers, as previously described.⁷ The left lung was inflated with 5 ml of 4% paraformaldehyde in phosphate-buffered saline and fixed overnight at 4° C. Parasagittal slices of the paraformaldehyde-fixed lungs were processed by standard methods and embedded in paraffin.¹² Serial 4 μ m sections were stained with hematoxylin and eosin. To document immunohistochemical evidence of RSV infection in the lung tissue sections of animals, a rabbit polyclonal, anti-RSV antibody (DAKO B344) was used to test for RSV antigens on paraformaldehyde-fixed, paraffin-embedded lung sections, according to our previously described immunoperoxidase reaction.⁷ RSV lung infection was considered present when a positive result (i.e., cytopathic effect of syncytial formation in culture or specific

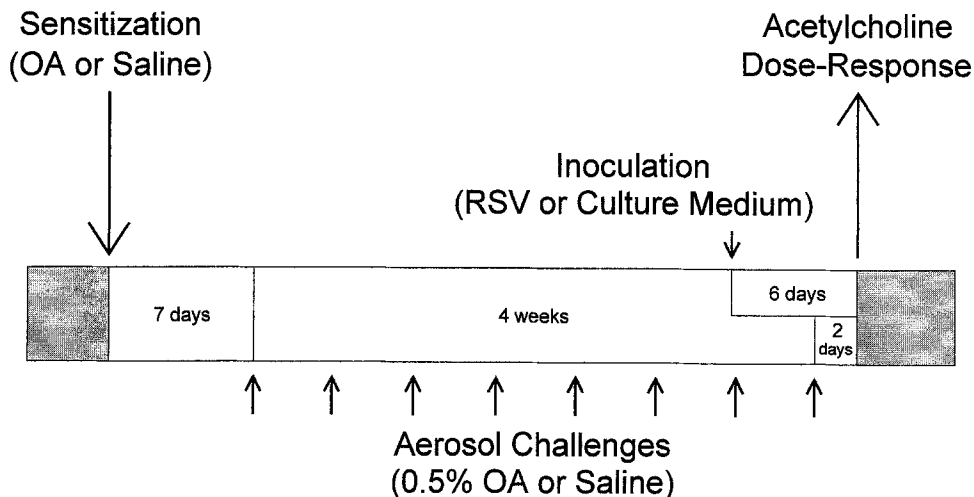


FIG. 1. Schematic representation of the time course of sensitization and viral inoculation of guinea pigs. OA, Ovalbumin.

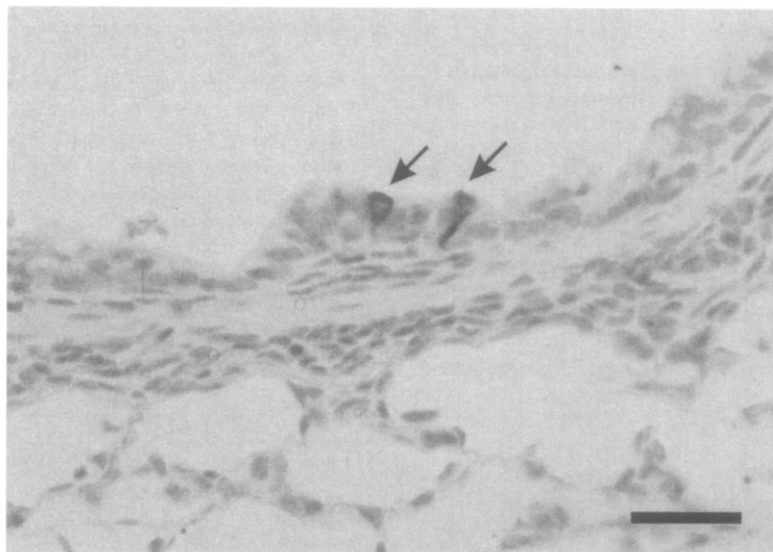


FIG. 2. Photomicrograph of airway from an RSV-inoculated guinea pig, showing positive immunostaining for viral antigens in cytoplasm of cells within airway epithelium (arrows). (Hematoxylin counterstain; scale bar represents 50 μ m).

cytoplasmic immunostaining with anti-RSV antibody [Fig. 2]) was observed with culture or immunohistochemistry.

Histologic scoring for airway inflammation

Membranous bronchioles were evaluated for epithelial necrosis, mononuclear cell infiltrates, granulocyte (mixed neutrophil-eosinophil) infiltrates, airway wall edema, hyperplasia of bronchus-associated lymphoid tissue (BALT), and goblet cell hyperplasia by using a previously described, highly reproducible, semiquantitative scoring method.⁷ Slides were coded so that the microscopist was not aware of the ovalbumin or RSV exposure status of the animal whose lung tissue was being examined. For a given airway, each of the six histologic features was scored from 0 (normal) to 2 (moderate to severe changes) by comparison with standard photomicrographs of guinea pig airways. Ten membranous bronchioles throughout the lung

were examined per animal, and the observed score for each histologic feature was expressed as the sum of individual airway scores, with a maximum score of $2 \times 10 = 20$ per feature per animal. A given airway was scored only once, and adjacent cuts of previously scored airways were avoided.

Statistical analysis

Statistical analyses were performed by using SYSTAT version 6 (SYSTAT Corporation, Evanston, Ill.). The mean R_L values for each acetylcholine dose were plotted against the log concentration of acetylcholine. R_L values at each acetylcholine concentration were compared between groups by using multivariate repeated-measures analysis of variance. Because interaction was observed between the groups, responses at each acetylcholine dose were compared by using an unpaired t test with Bonferroni's correction for multiple comparisons. A two-

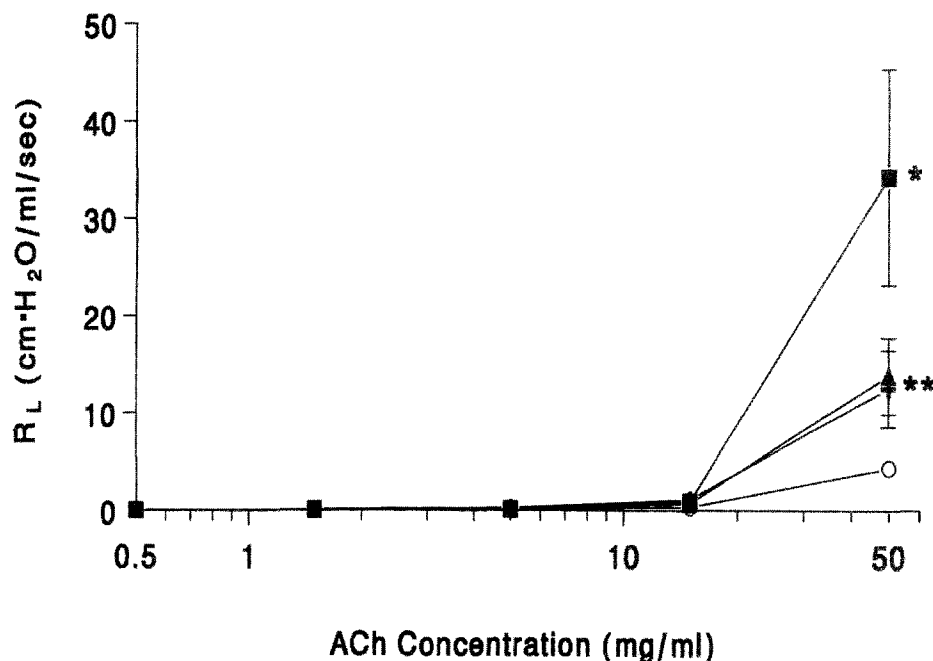


FIG. 3. Pulmonary resistance (R_L) changes in response to inhaled acetylcholine. Ovalbumin-, RSV- group (open circles); ovalbumin+, RSV- group (filled triangles); ovalbumin-, RSV+ group (plus signs); and ovalbumin+, RSV+ group (filled squares). * $p < 0.05$; ovalbumin+, RSV+ versus all other groups; ** $p < 0.01$: ovalbumin+, RSV- and ovalbumin-, RSV+ groups versus control ovalbumin-, RSV- group. ACh, Acetylcholine.

way analysis of variance with a sequential rejective Bonferroni procedure was used to compare histologic scores between the four groups of guinea pigs, at a sequential p value of less than 0.0083 (0.05/6), less than 0.01 (0.05/5), etc., until all six features had been analyzed.¹³

RESULTS

Of the 40 guinea pigs that were initially randomized into the four study groups, three died during the measurement of airway reactivity by acetylcholine challenge, two died during an ovalbumin challenge, and two RSV-inoculated animals were found to have no evidence of viral infection by either culture or immunohistochemistry. Results are therefore available for nine ovalbumin-, RSV- animals; eight ovalbumin+, RSV- animals; eight ovalbumin-, RSV+ animals, and eight ovalbumin+, RSV+ animals.

Acetylcholine challenge

Fig. 3 shows the results of changes in R_L to inhaled ACh in the four groups of animals. Compared with ovalbumin-, RSV- controls ($R_L = 4.3 \pm 1.1$ cm H₂O/ml/sec [mean $\pm$ SEM]), either allergen sensitization or RSV infection alone increased maximal responses to acetylcholine (13.7 ± 3.9 and 12.4 ± 3.9 cm H₂O/mL/sec, respectively) ($p < 0.05$). Animals from the ovalbumin+, RSV+ group had significantly higher R_L to inhaled acetylcholine at 50 mg/ml (34.0 ± 11.0 H₂O/mL/sec) than animals from the ovalbumin-, RSV+ group and the ovalbumin+, RSV- group ($p < 0.05$).

Histologic scoring

Representative airway histologic features from the four groups of animals are shown in Fig. 4. Fig. 5 shows the results of histologic scoring of guinea pig airways. In comparison with the ovalbumin- RSV- control animals, the ovalbumin+, RSV- group had significantly increased scores for airway epithelial necrosis, mononuclear and granulocyte infiltrates, airway wall edema, BALT hyperplasia, and goblet cell metaplasia. The ovalbumin-, RSV+ group had significantly increased scores for airway epithelial necrosis and mononuclear and granulocyte infiltrates. The ovalbumin+, RSV+ group had significantly increased scores for epithelial necrosis in comparison with the ovalbumin+, RSV- group ($p < 0.01$), with no significant differences observed between these groups in the other five histologic features examined.

DISCUSSION

The results of this study show that a preexisting allergic state, which includes increased nonspecific airway reactivity to acetylcholine challenge and airway inflammation, exacerbates the increase in airway reactivity during acute RSV bronchiolitis in the guinea pig. This virus-induced potentiation of airway reactivity is associated histologically with changes indicative of a combined effect of those observed with either ovalbumin or RSV alone. These observations are in agreement with the in vitro finding of increased tracheal responsiveness of ovalbumin-sensitized animals

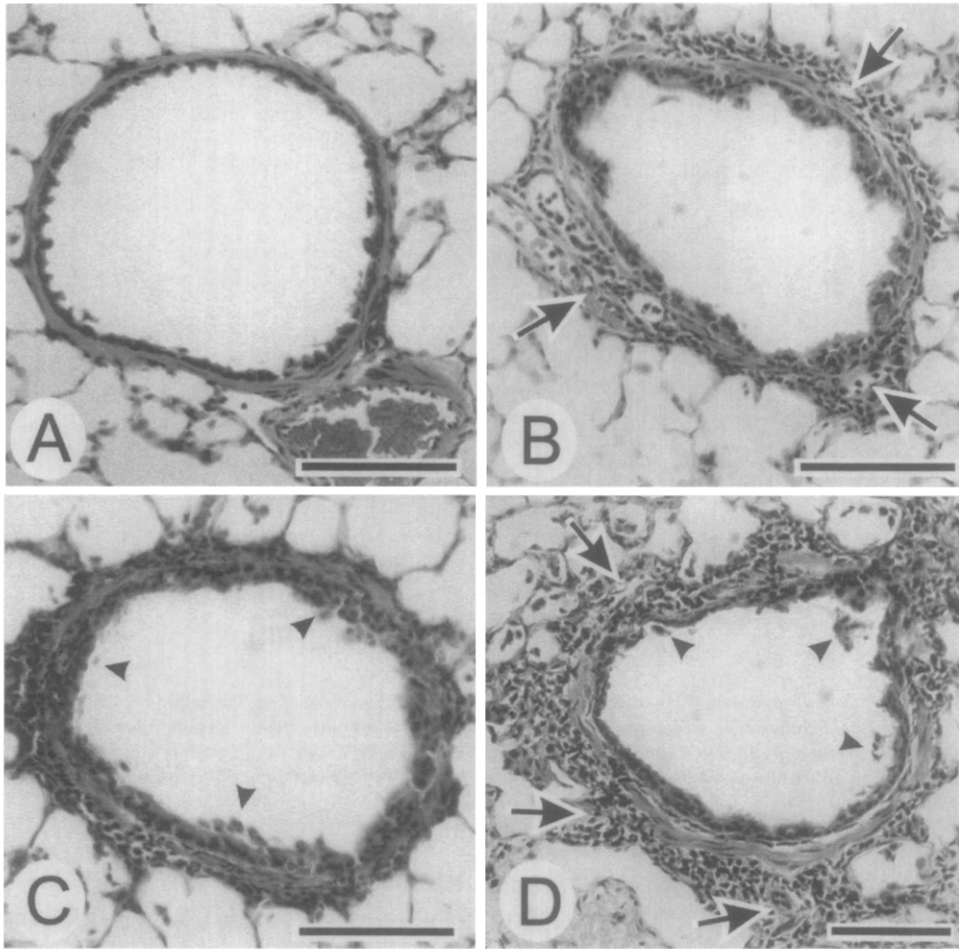


FIG. 4. Photomicrographs showing representative airway histologic features in ovalbumin-, RSV- controls (**A**), ovalbumin+, RSV- animals (**B**), ovalbumin-, RSV+ animals (**C**), and ovalbumin+, RSV+ animals (**D**). **A**, Near-normal membranous bronchiole (center) and associated pulmonary artery (bottom right). **B**, Peribronchiolar inflammatory cell infiltrate, airway wall edema, and congested adventitial blood vessels (arrows) in an ovalbumin-sensitized animal. **C**, Mild necrosis and sloughing of bronchiolar epithelial cells (arrowheads) in an RSV-infected animal. Note that peribronchiolar inflammation does not show prominent airway wall edema. **D**, Features suggestive of combined effects of ovalbumin sensitization and RSV infection in which inflammatory cell infiltration is associated with considerable airway wall edema (arrows) and epithelial necrosis (arrowheads). (Hematoxylin and eosin staining; scale bar represents 250 μ m).

to acute viral infection.⁵ Our observations of virus-induced increases in epithelial necrosis in the ovalbumin+, RSV+ animals when compared with the ovalbumin+, RSV- animals provide new information about possible mechanisms of the exaggerated response to viral respiratory infections observed in allergic individuals. Although Fryer and Jacoby¹⁴ have reported a decrease in the M2 muscarinic rereceptor-mediated inhibition of acetylcholine release from parasympathetic nerves in the lungs of guinea pigs infected with parainfluenza type 3 virus, such a mechanism could not explain the hyperresponsiveness to exogenous acetylcholine that we observed.

The significantly increased airway reactivity to acetylcholine in association with significantly increased airway epithelial necrosis and mononuclear cell and granulo-

cyte infiltrates in the ovalbumin-, RSV+ guinea pigs confirm our previous observations in this animal model of acute bronchiolitis.^{7,8} In addition, our findings with the histologic scoring system extend previous observations in ovalbumin-sensitized guinea pigs⁹ by documenting that ovalbumin sensitization augments several histologic features of airway inflammation, including mononuclear and granulocyte infiltration, airway wall edema, BALT hyperplasia, and goblet cell metaplasia. Which of the multiple changes we have noted is responsible for the increase in airway responsiveness is impossible to determine. However, it is probable that our results are due to combined effects. For example, airway wall edema itself would accentuate any changes produced by inflammatory cells. It should be noted that the histologic scoring system, although convenient to use and highly reproducible between

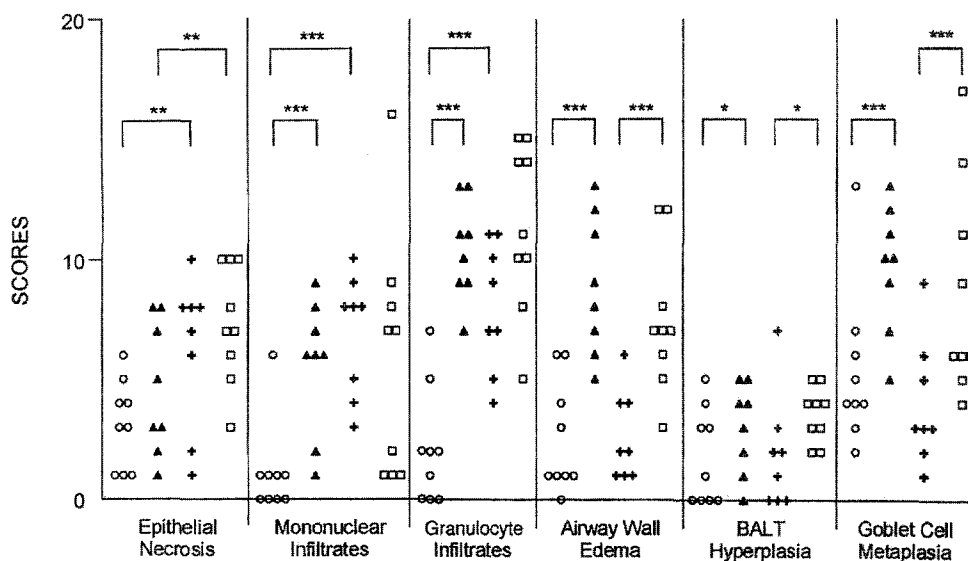


FIG. 5. Histologic scoring of airways from ovalbumin-, RSV- controls (open circles); ovalbumin+, RSV- group (filled triangles); ovalbumin-, RSV+ group (plus signs); and ovalbumin+, RSV+ group (open squares). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

and within observers,⁷ cannot distinguish the phenotypes of inflammatory cells (e.g., lymphocyte subsets) that comprise airway wall infiltrates, and this is further limited by the relative paucity of immunologic reagents available for the study of inflammatory responses in guinea pigs.

There are several mechanisms by which virus-induced increases in airway epithelial cell necrosis observed in the ovalbumin+, RSV+ animals could potentially augment airway responsiveness to acetylcholine. For example, loss of ciliated epithelial cells and the inflammatory response to viral infection would decrease the effectiveness of mucociliary clearance, while also increasing the amount of intraluminal mucus and cellular debris that must be removed from the tracheobronchial tree. In addition, increased epithelial cell lysis could result in decreased production of bronchodilating substances such as prostaglandin E_2 ,¹⁵ which modulates airway smooth muscle tone. Virus-induced epithelial cell lysis could also potentiate effects of a variety of bronchoconstricting neuropeptides such as substance P and neurokinin A, because epithelial cells contain high concentrations of neutral endopeptidase (enkephalinase), which metabolizes these peptides,^{16,17} and inhibition of neutral endopeptidases by agents such as toluene diisocyanate enhances the bronchoconstrictor effects of substance P.¹⁸ Alternatively, virus-induced epithelial necrosis could result in increased permeability and production of inflammatory edema in the airways, a process shown to be dependent on neurokinins.¹⁹ Which of these potential mechanisms of enhanced airway responsiveness from virus-induced airway epithelial necrosis is of primary importance requires further investigation.

In summary, this study has shown that in the presence

of a preexisting allergic state, RSV infection produces an exaggerated increase in airway reactivity that is associated with histologic evidence of increased epithelial damage when compared with the effects of ovalbumin sensitization or RSV infection alone. Children who have acute bronchiolitis may represent a subpopulation of individuals whose severe clinical manifestations of RSV infection represent a "marker" for an underlying allergic state.

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