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Viral Bronchiolitis During Early Life Induces Increased Numbers of Bronchiolar Mast Cells and Airway Hyperresponsiveness

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The objectives of this study were to determine the kinetics of Sendai virus-induced increases in bronchiolar mast cells and to determine whether virus-induced increases in bronchiolar mast cells were associated with increased airway responsiveness to methacholine and with altered allergic inflammatory responses to antigen stimulation. Mast cell density in intrapulmonary airways was measured in outbred CD (Crl:CDBR) rats by use of morphometric techniques at 7, 15, 30, 60, and 90 days after viral or sham inoculation. Density of bronchiolar mast cells was higher in virus-inoculated rats than in control rats at 30, 60, and 90 days after inoculation ($P < 0.01$), but not at 7 or 15 days after inoculation. Total pulmonary mast cell numbers were increased in virus-inoculated rats at 30 days after inoculation. Rats at 42 days after viral inoculation had over a threefold increase in sensitivity to the concentration of nebulized methacholine that would stimulate a 50% increase in respiratory resistance. Virus-inoculated rats sensitized to ovalbumin had over a 10-fold increase ($P < 0.02$) in pulmonary neutrophils that were recovered by bronchoalveolar lavage at 4 hours after ovalbumin aerosol challenge. Virus-inoculated rats at this time also had higher densities of neutrophils in bronchiolar walls than allergen-exposed control rats. The results indicate that Sendai virus induces increases in numbers of bronchiolar mast cells at times from 30 to 90 days after inoculation, and that mast cell increases are associated with airway hyperresponsiveness to methacholine and heightened allergic airway inflammatory reactions. (Am J Pathol 1990, 137:821–831)

Viral bronchiolitis and pneumonia are common diseases of childhood that result most often from infection by respiratory syncytial virus, parainfluenza viruses, adenoviruses, and rhinovirus.[1–6] Sequelae of viral bronchiolitis and pneumonia that have been described include persistent increases in airway resistance, recurrent wheezing or airway hyperresponsiveness, and other forms of respiratory disease in later life. Airway hyperresponsiveness is characterized by heightened sensitivity or exaggerated bronchoconstriction to airway stimulants such as cold air, methacholine, or histamine. Children younger than 2 years old have the greatest risk of sequelae from viral bronchiolitis,[1,4] and pathogenetic mechanisms that have been proposed as being important in this enhanced susceptibility of infants include immunologic and physiologic immaturity, small airway size, and the rapid rate of postnatal lung growth and remodeling.[1,4,6,7]

A variety of animal models have been developed for studying effects of viral respiratory disease on lung structure and function and airway hyperresponsiveness.[7–16] We have been studying parainfluenza type 1 (Sendai) virus infection in neonatal rats as an experimental model of virus-induced injury during early life[7,14,17–19] and have demonstrated that neonatal bronchiolitis and pneumonia induced by this virus results in bronchiolar hypoplasia and alveolar dysplasia that is not compensated for by additional postnatal lung growth.[7] Bronchiolar hypoplasia and alveolar dysplasia induced by this infection is associated with persistent increases in total lung resistance. Neonatal Sendai virus infection during early life also induces persistent alterations in pulmonary inflammatory cells. Rats inoculated with Sendai virus at 5 days of age were previously reported to have increased numbers of bronchio-

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lar mast cells and pulmonary eosinophils 90 days after inoculation.[19]

The objectives of this study were to determine the kinetics of parainfluenza virus-induced increases in bronchiolar mast cells and to assess whether virus-induced increases in pulmonary mast cells are associated with heightened airway responsiveness to a cholinergic agonist (methacholine) and to airway allergen challenge after active sensitization of bronchiolar mast cells with homocytotropic (IgE) antibody.

Materials and Methods

Experimental Design

Four experiments were conducted on outbred rats inoculated with virus or saline at 5 days of age. In the first and second experiments, the objective was to quantitate alterations in numbers of airway and total lung mast cells after viral bronchiolitis. In the first experiment, lungs were fixed from eight virus-inoculated and eight control rats at 7, 15, 30, and 60 days after inoculation. Eight control rats were studied on the day of inoculation (0 days). The lungs from four of the eight rats were fixed with Karnovsky's fixative, and the lungs from the four other rats were fixed with Carnoy's fixative containing chloroform. Four control rats were studied on the day of inoculation (0 days). The lungs were fixed with Karnovsky's fixative. An additional 22 rats were studied by the use of elastase digestion techniques to quantitate total pulmonary mast cells. In the second experiment, four infected and control rats each were inoculated with virus or saline, and mast cell densities were determined 90 days later from lungs fixed in Karnovsky's fixative.

In the third experiment, the objective was to evaluate the capacity of bronchiolar mast cells to mediate pulmonary recruitment of inflammatory cells. Rats that were virus- or sham-inoculated were actively sensitized to ovalbumin or injected with saline 30 days after inoculation. Rats were exposed to nebulized ovalbumin 12 days later. Four to eighteen hours later, either rats were studied by pulmonary lavage or their lungs were fixed for histology and electron microscopy. Three to six rats were studied in each group.

In the fourth experiment, five virus-inoculated and four sham-inoculated rats were evaluated for airway responsiveness to methacholine at 42 days after inoculation.

Rats and Viral Inoculation

The details of the rat inoculations and viral procedures for this study have been described.[7,17] Briefly, pregnant female outbred rats, Crl:CDBR (Charles River Breeding Laboratories, Inc., Portage, MI) were purchased at 14 days of gestation. The pregnant dams for control and experimental groups were separated and housed in adjacent isolation rooms that were individually ventilated. Room temperature was maintained at 70° to 72°F, and relative humidity was maintained between 50% and 55%. Rats were free of spontaneous pulmonary lesions except for changes that were associated with experimental Sendai virus infection. Rats were free of serum antibodies to pneumonia virus of mice, mycoplasma, and sialodacryoadenitis virus/rat coronavirus as detected by enzyme-linked immunosorbent assay conducted by the Research Animal Resource Center, University of Wisconsin-Madison. Control rats remained seronegative for Sendai virus. Newborn (neonatal) rats were not disturbed until they were inoculated at 5 days of age.

At 5 days of age, rats in virus groups were exposed to parainfluenza type 1 (Sendai) virus at a concentration of 1 to 2 plaque-forming units (PFU) of virus per milliliter of gas in a Tri-R aerosol exposure apparatus (Tri-R Instruments, Rockville Centre, NY) for 15 minutes. Control rats were exposed to saline. Viral assays were performed as described previously to calibrate exposure.[7,17] Rats in experiments 2, 3, and 4 were inoculated with strain P3193 Sendai virus, which had been produced in embryonated chicken eggs. Rats in experiment 1 were inoculated with strain P3193-C3A, which had been selected by repeated plaque purifications in Madin-Darby bovine kidney cells. Lungs from three rats per experimental group were examined by histologic methods as described below at 7 days after inoculation to confirm successful infection and induction of necrotizing bronchiolitis and interstitial pneumonia. Rats were weaned at 21 days of age and separated into groups of males and females.

Necropsy and Lung Preparation for Histology and Electron Microscopy

Rats were examined at various times from 0 to 90 days after viral inoculation. Rats for morphologic studies were anesthetized with sodium pentobarbital and killed by exsanguination and pneumothorax. The lungs were prepared for light microscopy as previously described.[7,17] Briefly, lungs were fixed by tracheal perfusion at 30 cm pressure with glutaraldehyde-paraformaldehyde fixative with cacodylate buffer (Karnovsky's fixative) or with Carnoy's fixative. Four transverse sections were taken of the fixed left lung for histology. The first section was taken at the level of the hilus through the lobar bronchiole. The second section was taken between the hilar section and the cranialmost margin of the lung. The third and fourth

sections were taken at points equidistant caudal from the hilar section and cranial from the caudal border. An additional section was taken through the mainstem bronchi slightly distal to the carina. Tissue blocks were embedded in paraffin for preparation of hematoxylin and eosin (H&E)-stained sections cut at 6 μ.

Samples of the right middle lung lobe from aldehyde-fixed lungs were prepared for transmission electron microscopy as described.[7,17] Briefly, tissue blocks were dehydrated in acetone and embedded in a mixture of Araldite 5002 and Embed 812 (Electron Microscopy Science, Fort Washington, PA). Large 1.5-μ sections were cut with glass knives and stained with methylene blue and azure II. Sections were examined, and selected areas were remounted on plastic sections and ultrathin sections were cut. Ultrathin sections were stained with lead citrate and uranyl acetate.

Mast Cell Quantitation in Tissue

Initially we compared bronchiolar mast cell densities in sections of lung fixed with Carnoy's fixative with chloroform[20] and with Karnovsky's fixative[7] by staining the sections as follows: 1) with 0.1% toluidine blue in 0.01 mol/l (molar) acetate buffer, pH 2.5; 2) with Wolbach's Giemsa stain[20]; and 3) with 1% alcian blue in 0.5% HCl (pH 0.5).[21] We found that bronchiolar and alveolar mast cells in infected and control rats stained in a closely comparable manner with toluidine blue, Giemsa stains, or alcian blue after fixation of lungs with Karnovsky's or Carnoy's fixative. Lungs from virus-inoculated rats from a separate experiment that were fixed with Carnoy's fixative (n = 4) and with Karnovsky's fixative (n = 3 or 4) at 30 days after inoculation were compared for mast cell densities as described below. Carnoy-fixed tissues had the following bronchiolar mast cell densities (mean ± standard deviation [SD]) with each stain: toluidine blue, 1.57 ± 1.17; Giemsa, 0.90 ± 0.16; and alcian blue, 1.91 ± 0.15 mast cells/mm wall. Aldehyde-fixed tissues had the following bronchiolar mast cell densities: toluidine blue, 0.90 ± 0.64; and Giemsa, 1.69 ± 0.72 mast cells/mm wall. There were no significant differences among the means by analysis of variance.

Because fixation in Karnovsky's fixative allowed combined analysis of lungs by light and transmission electron microscopy as well as mast cell quantitation when Giemsa stains were used, we focused quantitative studies in experiments 1 and 2 on paraffin sections stained with Wolbach's Giemsa stain from lungs fixed with Karnovsky's fixative.

Mast cells were counted in sequential fields of mainstem bronchi, of lobar bronchioles, and of terminal bronchioles at a magnification of 400× as previously described.[19] All of the mainstem bronchi and lobar bronchi sections were sequentially scanned in 0.44-mm diameter intervals, and all of the mast cells in the airway lamina propria and mucosal epithelium in the field with epithelium in the center were counted. At least 30 fields of terminal bronchiole were counted per rat. Mean counts for all fields per airway type per rat were calculated.

Total pulmonary mast cell numbers per rat were determined in 10 control rats and 12 virus-inoculated rats at 30 days after inoculation using elastase dissociation of pulmonary mast cells as described previously.[19] Briefly, lungs were lavaged via the airways and perfused via the vasculature to remove blood cells and alveolar macrophages before enzymatic dissociation with elastase solution. Dispersed cells were recovered after filtration through gauze and nylon mesh filters. Total cell counts per lung were determined through the use of a hemacytometer, and mast cells were stained in cytocentrifuge preparations with Giemsa stain. Total numbers of mast cells per lung were calculated from the total cell recovery and differential mast cell values.

Ovalbumin Sensitization and Challenge

In experiment 3, rats were sensitized to ovalbumin 30 days after viral or sham inoculation as described.[22] Sensitized rats received a 1.0 ml subcutaneous suspension containing 1.0 mg of chicken ovalbumin, grade V (Sigma Chemical Company, St. Louis, MO), and 200 mg of $AL(OH)_3$, in physiologic saline. They also received 10^9 killed *Bordetella pertussis* in 0.5 ml aqueous solution (Difco, Detroit, MI) by intraperitoneal injection. This method of sensitization results in heat-labile passive cutaneous anaphylaxis titers of 1:8 to 1:64, and precipitating antibody to ovalbumin cannot be detected by radial immunodiffusion.[22] Nonsensitized rats received subcutaneous and intraperitoneal saline injections.

Twelve days after sensitization or saline injection, rats were challenged for 30 minutes to 1.0 mg ovalbumin/ml aqueous solution nebulized with a model 40 nebulizer (DeVilbiss, Somerset, PA) connected to an electric pump generating 7 psi pressure. At the end of the exposure period, rats (n = 3 per group) were returned to their cages and studied at 4 hours or at 12 hours later by pulmonary histology. One sensitized rat and one nonsensitized control rat were challenged with ovalbumin and then immediately killed and prepared for pulmonary histology and electron microscopy to determine whether ovalbumin caused degranulation of mast cells in sensitized, but not in nonsensitized rats. Additional control and virus-inocu-

lated rats (n = 5 or 6 per group) were studied by pulmonary lavage at 4 and 18 hours after ovalbumin challenge.

Serum was tested from sensitized control and virus-inoculated rats, and they had comparable titers of homologous heat-labile, 48-hour passive cutaneous anaphylaxis activity characteristic of reactions mediated by IgE antibody.[22]

Histologic sections for experiment 3 were prepared as described in the preceding section and were stained with Giemsa stain to allow quantitation of inflammatory cells, including eosinophils, in bronchiolar walls. Terminal bronchiolar inflammatory cells were quantitated in tissue sections at 4 hours after exposure to nebulized ovalbumin so that we could determine whether virus-induced increases in pulmonary mast cells were associated with enhanced inflammatory cell recruitment 4 hours after homocytotropic-antibody–mediated mast cell degranulation and secretion.

Histologic sections stained with Giemsa stain from each rat were scanned at 200× magnification, and each terminal bronchiole generation in each of the four sections was characterized as either having peribronchiolar aggregates of neutrophils and eosinophils or being devoid of them. Percent airways with polymorphonuclear cell aggregates (ie, neutrophils and eosinophils) was calculated by dividing numbers of neutrophil- and eosinophil-positive airways by the total number of terminal bronchioles examined per rat. Percent airways with peribronchiolar aggregates of lymphocytes and macrophages were determined by a similar method of examination. Density of eosinophils and neutrophils per millimeter length of bronchiolar wall were determined by counting numbers of eosinophils or neutrophils per field at a magnification of 1000× in eosinophil- and neutrophil-positive airways. In each field examined, epithelial cells were centered in the field, and all the neutrophils or eosinophils were counted per 0.178-mm diameter field. Twenty fields were counted per rat unless the animal had insufficient numbers of eosinophil- and neutrophil-positive airways. The counting procedure was randomized by starting with the lung section in the top right-hand corner of the slide and proceeding in an s-shaped pattern to examine each section until 20 fields in bronchioles were counted. Lung blocks were arranged in paraffin blocks, and sections were arranged on the slide in an arbitrary manner, which facilitated a random starting point in the systematic sampling method.

Bronchoalveolar Lavage

After rats were anesthetized and exsanguinated, the trachea was cannulated, and the lungs were inflated with phosphate-buffered saline (PBS) until they filled the thoracic cavity. The saline was withdrawn with a syringe. The lavage was repeated a total of five times. The saline infused and volume recovered were recorded. Total cell counts were made with a hemacytometer. Cytocentrifuge cell samples of lavage fluid were made with a Cytospin 2 (Shandon Southern Instruments, Inc, Swickley, PA) and stained with Giemsa solution. A cell differential was calculated after counting 500 stained cells. Total cell numbers recovered per lung were calculated (macrophages, neutrophils, lymphocytes, epithelial cells, and eosinophils).

Methacholine Airway Responsiveness

Rats were anesthetized with urethane 1.0 g/kg intraperitoneally and received a total of up to 1.2 g/kg during the course of the experiments. Endotracheal tubes prepared from 8-Fr feeding tubes were inserted through a cervical tracheostomy and secured with silk ligature. A rodent plethysmograph (Hans Rudolf, Kansas City, MO), modified for use with intubated rats, was used to measure changes in thoracic volume. The interior of the body box was filled with copper sponges to maintain mostly isothermic gas compression conditions at the rates used for the experiments. Plethysmograph pressure was measured with a Validyne DP45-14 transducer (Validyne Engineering Corp., Northridge CA), referenced to an insulated gallon bottle with a slow leak to the room. There was also a slow leak between the plethysmograph and the reference bottle to stabilize thermal drifts. The plethysmograph was calibrated with a syringe, injecting air at rates within the range encountered in the experiments. A plastic bag containing water simulated the rat's displacement volume. Lung inflation pressure was monitored at the connector between the endotracheal tube and a Harvard rodent ventilator using a Motorola MPXIIDP differential pressure transducer (Motorola Semiconductors, Phoenix, AZ) referenced to the plethysmograph interior. Amplified signals from the transducers were fed to a pulmonary mechanics analyzer (Buxco Model 6, Sharon, CT) that computed resistance for each breath using isovolume points at 55% tidal volume. The flow-specific resistance of the tracheal tube and connectors was subtracted from measured resistance to obtain respiratory system resistance. Transducer signals were in phase to at least 7 Hz.

Pancuronium-paralyzed, ventilated rats were used for the methacholine responses. Methacholine was nebulized with an ultrasonic nebulizer with interchangeable plastic cups (DeVilbiss model 35A). Forty milliliters of solution was placed in each cup, and concentrations of 0.5, 1, 2, 4, 8, 16, and 32 mg/ml were prepared. A second Harvard rodent ventilator was used to pass ventilations through the nebulizer cups at a tidal volume of 2.5 ml and frequency of 80 ventilations per minute. A cup was placed on the nebulizer with the ventilator on for about 2 minutes

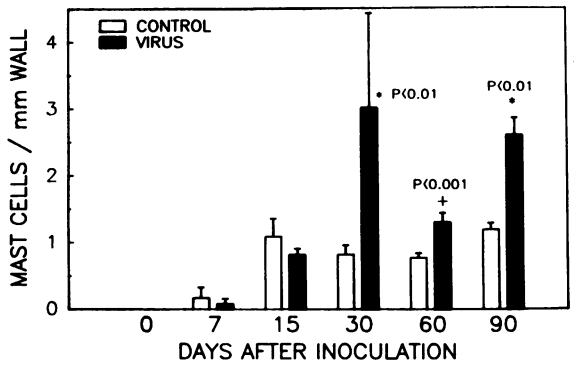

Figure 1. *Density of mast cells in bronchioles of rats inoculated with virus or saline (control) at 5 days of age. Group means (n = 4 per group) and standard error bars. There are significant (P < 0.05) increases in terminal bronchiolar mast cell densities with age. Significant differences determined between means evaluated by 2-way ANOVA and Tukey test. Means between virus and control groups differ at 30, 60, and 90 days.*

before a challenge to assure that a steady state of aerosol output had been reached. Challenges were done by removing the main ventilator from the connector port, and plugging in the aerosol ventilator for 10 breaths, then switching back to the main ventilator. The response to methacholine was taken as the highest average resistance occurring in consecutive 12-second averages in the first minute after completion of the breaths of aerosol. Subsequent challenges were done after a rat returned to baseline resistance.

On a graph of log methacholine *versus* log resistance, we plotted resistance immediately before challenge and peak resistance after challenge for each methacholine concentration. Then we determined graphically the concentration of methacholine at which the resistance on the response line was 150% of the resistance on the baseline. This concentration was defined as the provocative concentration 150% (PC150).

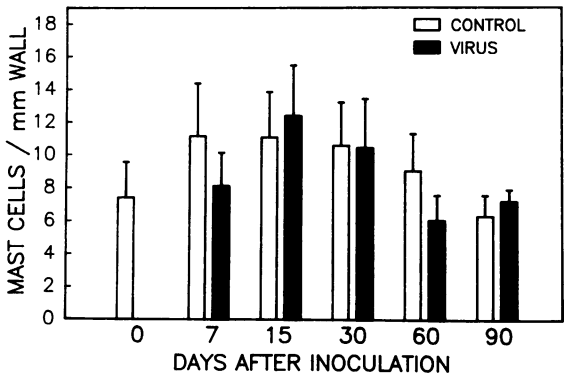

Figure 2. *Density of mast cells in main-stem bronchi. Group means (n = 4 per group) and standard error bars. There are no significant differences in mast cell densities with age. Significant differences determined between means evaluated by two-way ANOVA and Tukey test.*

Table 1. *Mast Cells Recovered from Elastase-dissociated Lung 30 Days After Inoculation: Pulmonary Mast Cells per Rat × 10^3*

Control (n = 10)	Virus (n = 12)
4.87 ± 6.32*	16.55 ± 2.90†

* Mean ± SD.
† $P < 0.05$, two-sided *t*-test.

Statistics

Mean values between groups were compared using two-way analysis of variance (ANOVA) using a microcomputer-based statistical program (Systat, version 4, Evanston, IL). Differences between individual means were compared by individual Tukey *post hoc* tests. Percentage data was subjected to arcsine transformation before analysis. A two-tailed *t*-test was used to evaluate differences between means for total mast cell numbers in elastase-dissociated lung after logarithmic transformation of data. Two-tailed *t*-tests for groups with unequal variances were used to compare means when Bartlett tests indicated unequal variances among groups. Differences between the groups for PC150 methacholine responsiveness were tested with the Wilcoxon-Mann-Whitney test.[23]

Results

Mast Cell Distribution

In control rats fixed with glutaraldehyde and paraformaldehyde, pulmonary mast cells were identified in Giemsa-stained and in toluidine blue-stained sections in lamina propria and in loose connective tissue of bronchioles and bronchi, in loose connective tissue around pulmonary arteries and veins, and in subpleural connective tissue. Mast cells were not identified in alveolar duct walls or in interalveolar septa of control rats. Similar distributions of mast cells were identified in virus-inoculated rats. However, in rats at times from 30 to 90 days after viral inoculation, mast cells also were found in interalveolar septa of abnormally large and distorted (dysplastic) alveoli in proximal acinar areas and in interalveolar septa immediately adjacent to terminal bronchioles. No difference in mast cell distribution or density could be demonstrated in bronchioles or in alveolar tissue in either control or virus-inoculated rats after pulmonary fixation in Carnoy's fixative and staining techniques with toluidine blue or alcian blue (data not shown).

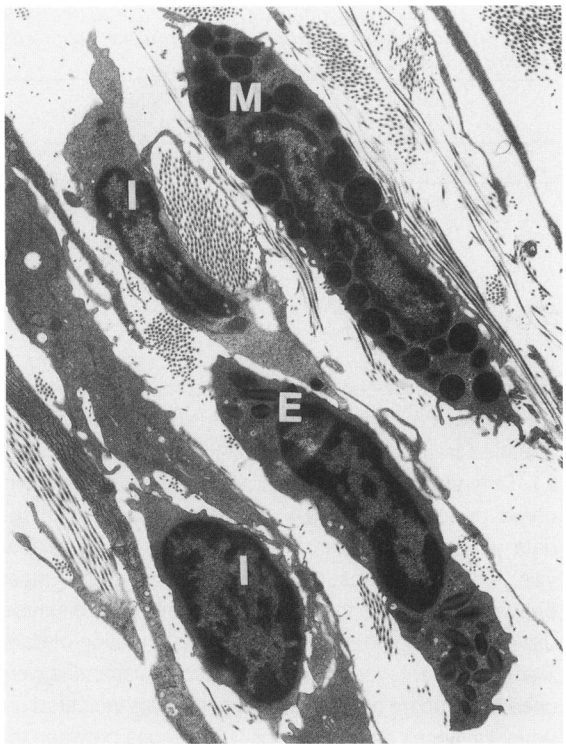

Figure 3. *Transmission electron micrograph of terminal bronchiolar lamina propria from a rat 30 days after Sendai virus inoculation. A mast cell (M), eosinophil (E), and two small mononuclear inflammatory cells (I) are located near collagen and fibroblast processes (×7,300).*

Airway Mast Cell Density

Mast cell density data for terminal bronchioles and mainstem bronchi are presented in Figures 1 and 2. In terminal bronchiolar wall (Figure 1), mast cells were rarely found until 7 days after inoculation. There were age-associated increases ($P < 0.05$) in bronchiolar mast cell density. Virus-induced increases in bronchiolar mast cell numbers were first detected at 30 days after viral inoculation when there was over a threefold increase in the density of mast cells as compared with age-matched controls. Mast cells in bronchiolar wall 30 days after viral inoculation had uniformly electron-dense granules, and the cells were associated with collagen, small mononuclear cells, and occasional eosinophils (Figure 3). Virus-inoculated rats also had significantly elevated numbers of bronchiolar mast cells at 60 and 90 days after inoculation (Figure 1). Total mast cell numbers recovered from elastase-dissociated lung at 30 days after inoculation were increased in virus-inoculated rats (Table 1).

At 30 days after inoculation, virus-inoculated rats also had increased numbers of bronchioles with aggregates of eosinophils and neutrophils in the lamina propria (Table 2). Eosinophil and neutrophil densities within these bronchiolar aggregates were not increased in virus-inoculated rats.

In mainstem bronchi (Figure 2) and in lobar bronchioles (data not shown), there were no significant differences in mast cell densities between virus-inoculated and control rats. There were also no age-associated changes in mast cell density in these larger airways.

Airway Response to Methacholine

Summary data from methacholine dose-response tests conducted at 42 days after inoculation are shown in Figure 4. The concentration of methacholine required to stimulate an increase in respiratory system resistance to 150% of baseline was significantly lower in virus-inoculated rats than in sham-inoculated control rats. This indicates that virus-inoculated rats have enhanced airway responsiveness (hyperresponsiveness) to methacholine, a cholinergic airway smooth muscle agonist.

Allergic Inflammatory Response

Degranulation of bronchiolar mast cells in rats that had been actively sensitized with ovalbumin was demonstrated after aerosol exposure to ovalbumin (Figure 5). Mast cell degranulation was not observed in rats exposed to ovalbumin that had not been previously sensitized.

Results from pulmonary lavage at 4 and 18 hours after sensitization and aerosol exposure are shown in Figure 6, and quantitative data on tissue inflammatory cell responses at 4 hours after ovalbumin aerosol are presented in Table 2. Aerosol exposure of ovalbumin in ovalbumin-sensitized rats resulted in significant increases in numbers of bronchioles with aggregates of eosinophils and

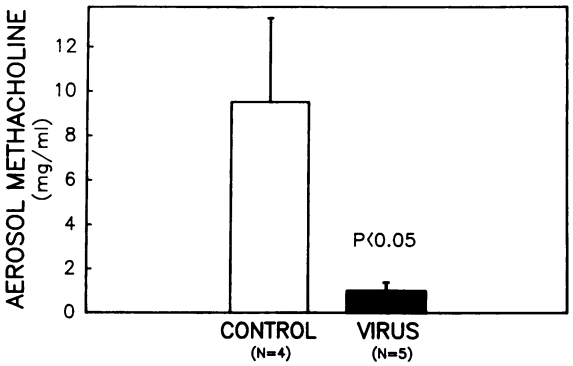

Figure 4. *Methacholine provocation test (PC150) of control and virus-inoculated rats at 42 days after inoculation. The test determines the concentration of methacholine nebulized that will result in an increase in respiratory system resistance to 150% of baseline. Mean ± SE.*

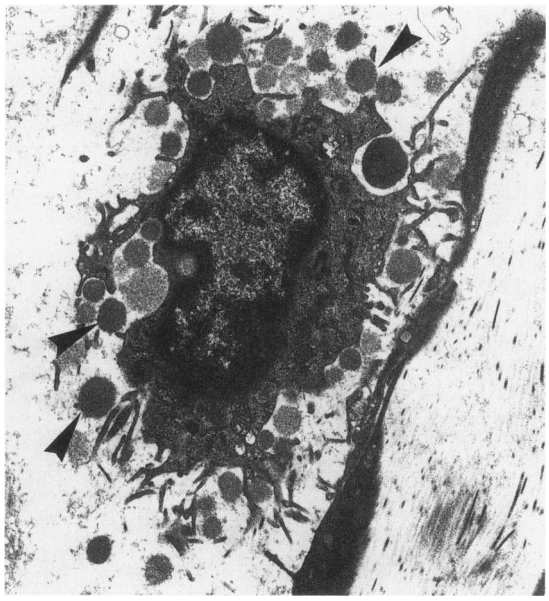

Figure 5. *Terminal bronchiolar mast cell from a ovalbumin-sensitized rat 42 days after virus inoculation that has been exposed to ovalbumin aerosol and studied within 5 minutes of the end of the exposure. There is fusion and extracellular release (arrowheads) of mast cell granules (×6500).*

neutrophils in virus-inoculated rats at 4 hours after ovalbumin exposure as compared with controls. There were significantly higher neutrophil densities in the bronchiolar inflammatory cell aggregates of virus-inoculated rats than in controls (Table 2). This increase was associated with a significantly greater number of neutrophils in pulmonary lavage fluid of virus-inoculated rats (Figure 6) at 4 hours after aerosol antigen challenge. No differences were detected in eosinophil concentration in lavage fluid (Figure 6) or in eosinophil densities in bronchiolar inflammatory cell aggregates (Table 2) between control and virus-inoculated rats. Ovalbumin aerosol alone in nonsensitized rats had no effect on pulmonary inflammatory cells in pulmonary lavage fluid or in tissue eosinophil and neutrophil densities (data not shown).

Peak numbers of neutrophils and eosinophils were detected in lavage fluid at 18 hours after antigen challenge (Figure 6) and in tissue sections at 12 hours after antigen challenge (Figures 7, 8; quantitative data not shown), but there were no significant differences in mean values between virus-inoculated and control rats.

Viral infection was associated with a significant increase in peribronchiolar aggregates of lymphocytes and macrophages (Table 2), but antigen sensitization and exposure had no effect on peribronchiolar lymphoid aggregates.

Discussion

This study extends previous observations[19] indicating that parainfluenza virus infection during early life increases numbers of pulmonary mast cells. The major conclusions to be drawn from these experiments are that viral bronchiolitis and pneumonia during early life in rats results in increased numbers of bronchiolar mast cells as early as 30 days after viral infection and that the increased numbers of mast cells are associated with enhanced airway responsiveness to methacholine and heightened bronchiolar inflammatory cell responses to antigen challenge that stimulates mast cell degranulation.

Heterogeneity of mast cell phenotype has been identified in humans and rodents.[24-27] In rodents, connective tissue mast cells and mucosal mast cells have been identified. Generally rat mast cells of the connective tissue phenotype are found in locations such as skin and peritoneal cavity.[24,25] They do not lose staining affinity to metachromatic dyes such as toluidine blue after aldehyde fixation. They contain rat mast cell protease I, and have a long tissue life compared with mucosal mast cells. Connective tissue mast cells respond to secretogogues such as substance P and compound 48/80.[24,25,27] In addition to histamine, they release prostaglandin D_2 as the major arachidonic acid metabolite.[24,27] In the normal rat lung, mast cells have been reported to be predominantly of the connective tissue phenotype and are located in airway walls, in peribronchiolar connective tissue, and in connective tissue around blood vessels and in subpleural areas.[19,28,29] After bleomycin-induced lung injury, connective tissue mast cells increase in alveolar areas of the lung where fibrosis can be demonstrated.[29,30]

Mast cells with staining and enzymatic profiles consistent with mucosal mast cells have been reported in trachea and bronchi of rats.[28,29] Mucosal mast cells have formalin-sensitive granules that lose their metachromatic staining properties after aldehyde fixation. The granules are rich in oversulfated chondroitin sulfate and stain well

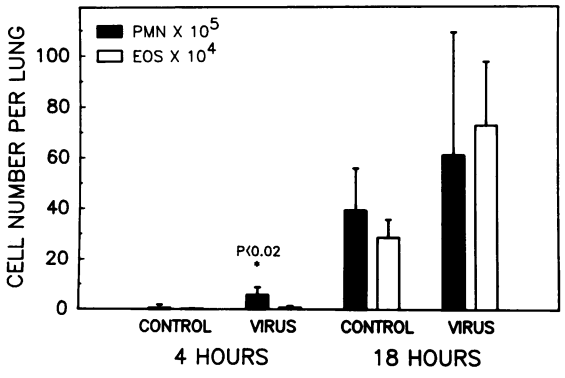

Figure 6. *Neutrophils and eosinophils recovered by pulmonary lavage at 4 and 18 hours after aerosol challenge with ovalbumin in control and virus-inoculated rats actively sensitized to ovalbumin. Mean ± SE (n = 5 or 6 per group). Virus-inoculated rats have increased numbers of neutrophils in lavage fluid at 4 hours (ANOVA and* post hoc *test.)*

Table 2. *Bronchiolar Inflammatory Cell Data 4 Hours After Ovalbumin Exposure*

	Eosinophil and neutrophil aggregates (% airways)	Eosinophil density (cells/mm wall)	Neutrophil density (cells/mm wall)	Lymphoid cell aggregates (% airways)
Nonsensitized				
Control	4.82 ± 1.10*	2.12 ± 0.49	3.31 ± 0.38	2.58 ± 1.84
Virus	14.09 ± 6.58#	3.69 ± 2.36	3.27 ± 1.32‡	5.64 ± 4.02
Sensitized				
Control	14.31 ± 3.67†	5.62 ± 1.21	6.27 ± 1.98‖	3.85 ± 2.06
Virus	38.77 ± 6.50†#	5.15 ± 3.74	22.18 ± 5.78‡‖	12.12 ± 4.56
Two-way ANOVA				
Effect of				
Virus	$P < 0.01$	NS	ANA	$P < 0.05$
Antigen sensitization	$P < 0.001$	NS	ANA	NS

* Mean ± SD. Each group n = 3. NS, no significant effect, 2-way ANOVA. ANA, ANOVA not applied because of unequal group variance by Bartlett test.
#† $P < 0.01$ 2-way ANOVA, Tukey test.
‡‖ $P < 0.05$ two-tailed *t*-test for groups with unequal variance.

with dyes such as alcian blue.[24] The granules retain their metachromasia after fixation in nonaldehyde fixatives such as Carnoy's fixative. Rat mucosal mast cells contain rat mast cell protease II and are not responsive to degranulating agents such as compound 48/80 and substance P. Mucosal mast cells in rats are reported to synthesize and release leukotriene C_4, rather than prostaglandins, as the principle arachidonic acid metabolite.[24,27] Increases in mucosal mast cells have been reported to be T-cell– or interleukin-3 (IL-3)–dependent, and murine mucosal mast cells are reported to have an approximately 40-day half-life.[24] Mucosal mast cells have not been reported to increase in number after experimental pulmonary injury with compounds such as bleomycin.[29,30]

The increased numbers of mast cells described in this study in bronchiolar walls as early as 30 days and extending at least until 90 days after viral inoculation stained well with toluidine blue after glutaraldehyde-formaldehyde fixation. This aldehyde insensitivity is consistent with the cells being of the connective tissue phenotype. Mast cells with comparable staining characteristics were present in areas of virus-induced alveolar distortion and enlargement (dysplasia) in proximal acinar areas. Mast cell densities in viral-infected rats were not increased in main-stem bronchi or in large caliber lobar bronchioles.

Increases in tissue mast cells have been reported in several experimental models, including bleomycin-induced pulmonary fibrosis and parasitic diseases.[27,29,30] Mast cell increases in tissues of rats and mice have been documented after *Nippostrongylus brasiliensis* infection, auto-anti-IgE response, and repeated injection with compound 48/80, polymyxin B, or nerve growth factor.[24,25,27,31] The ability of rats to respond to injury or stimuli with increases in tissue mast cells appears to be greater during early life than in adulthood.[24]

Experimental studies in rodents have identified several mechanisms that can lead to increases in mast cells in tissues. These include IL-3–dependent differentiation of

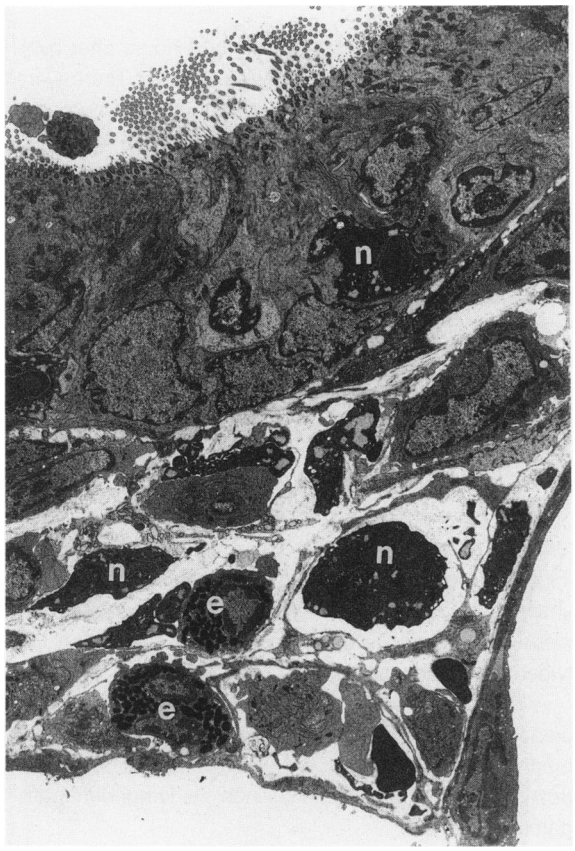

Figure 7. *Terminal bronchiole of virus-inoculated and ovalbumin-sensitized rat 18 hours after ovalbumin aerosol exposure. There is edema of the bronchiolar wall and neutrophils (n) and eosinophils (e) are present within the bronchiolar wall and between bronchiolar epithelial cells (×4300).*

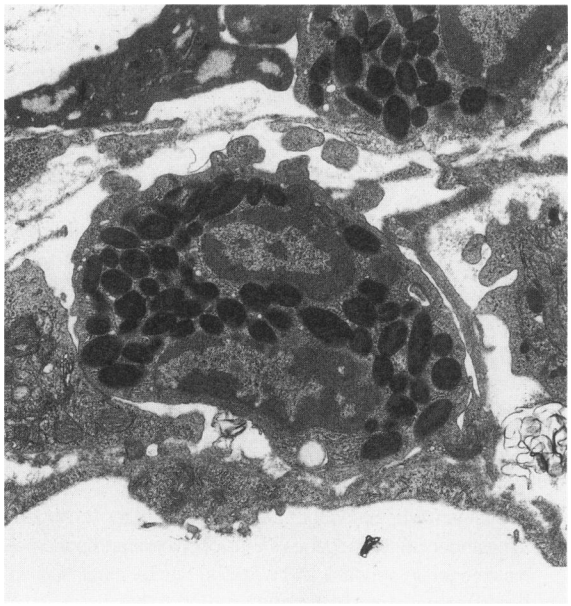

Figure 8. *Higher magnification of eosinophil in Figure 7 (×9000).*

bone marrow mast cell precursors, fibroblast-facilitation of mast cell precursor differentiation, and autocrine stimulation of mast cell proliferation and/or differentiation associated with mast cell degranulation.[24,25,32] There is evidence to indicate that both local proliferation of mature mast cells and tissue recruitment and differentiation of circulating mast cell precursors can contribute to expansion of tissue mast cell populations.[24]

Although the present study does not provide direct experimental evidence of specific mechanisms that might be important in the pathogenesis of virus-induced bronchiolar mast cell increases, there are several possible explanations. Sendai virus infection in rats induces severe necrotizing and proliferative bronchiolitis that is accompanied by lymphocyte infiltration.[7,17] The infection in neonatal rats leads to bronchiolar hypoplasia and multifocal mural bronchiolar fibrosis.[7] Recovery from Sendai virus infection in mice has been demonstrated to be T-cell–dependent,[33] and Sendai-virus–induced increases in bronchiolar mast cells could be secondary to T-cell infiltration into bronchiolar walls, with microenvironmental increased levels of IL-3 resulting in differentiation of mast cell precursors derived from blood or from pre-existing tissue precursors. Alternatively, mast cells may have been induced to differentiate from bronchiolar mast cell progenitors secondary to fibroblast proliferation and release of differentiation factors. Finally acute virus-induced inflammation may have resulted in bronchiolar mast cell degranulation with subsequent autocrine stimulation of mast cell proliferation or differentiation.[32] It has been demonstrated that mast cell lines synthesize and release IL-3 in response to degranulating stimuli,[34] which suggests that mast cell proliferation and differentiation might occur by a T-cell–independent mechanism, which may still be dependent on IL-3.

Mast cell increases were associated with airway responsiveness to methacholine and with enhanced recruitment of neutrophils into airways after active sensitization of pulmonary mast cells and antigen challenge. More detailed studies on airway hyperresponsiveness in rats after neonatal Sendai virus infection are the subject of a separate report.[35]

Airway hyperresponsiveness, or exaggerated bronchoconstriction to nonspecific stimulation such as cold air, histamine, or methacholine, has been linked to severe bronchiolitis during early life in children,[3–5,36] and it is reasonable to speculate that mast cell hyperplasia induced by viral airway injury during early life could be an important mechanism in the pathogenesis of persistent virus-induced airway hyperresponsiveness. The precise role of mast cells in airway hyperresponsiveness and asthma is poorly defined at this time.[37,38] However, several lines of experimental evidence indicate that mast cell mediators can contribute to airway hyperresponsiveness.

Lung mast cells in humans[24,39] and connective tissue mast cells in rats[24] synthesize and release prostaglandin D_2 (PGD_2), which has been identified as enhancing airway responsiveness to methacholine-induced bronchoconstriction in humans.[39] Neutral proteases released from mast cells, including tryptase and chymase, can alter airway function.[40,41] Most notably, mast cell tryptase increases bronchial responsiveness to histamine.[42] Mast cells also release chemotactic factors for neutrophils and eosinophils.[37] Neutrophils and eosinophils recruited secondary to release of chemotactic factors associated with bronchiolar mast cell hyperplasia could release other important molecules enhancing airway responsiveness, such as platelet-activating factor.[38]

Our initial studies aimed at evaluating mast cell function in rats with virus-induced increases in bronchiolar mast cells demonstrated that active sensitization of rats with ovalbumin resulted in bronchiolar mast cell degranulation when the rats were subsequently challenged by aerosol with ovalbumin. Control and viral-infected rats had comparable heat-labile passive cutaneous anaphylaxis titers, indicating that differences in IgE antibody response were not likely to be responsible for the virus-associated increases in bronchiolar neutrophil and eosinophil aggregates and in neutrophil numbers recovered by pulmonary lavage at 4 hours after antigen challenge. Release of chemotactic substances for eosinophils and neutrophils from increased numbers of bronchiolar mast cells could explain the virus-associated increase in polymorphonuclear cell response to antigen. Other mechanisms such as differing responses of complement-fixing antibody or of

lymphocytes to ovalbumin by virus-inoculated and control rats could also explain the differences in inflammatory cell responses. Although additional experimental studies will be required, this initial study provides encouraging results that suggest to us that virus-induced increases in bronchiolar mast cells could be functionally significant in promoting airway inflammation and hyperresponsiveness.

It is currently controversial whether mast cells or other inflammatory cells such as eosinophils and macrophages play a central role in the pathogenesis of airway hyperresponsiveness in human asthma.[37,38] Alternatively neural mechanisms and neuropeptide metabolism via enzymes such as neutral endopeptidase may play an equally or more important role.[38] This report describes a rat model of airway hyperresponsiveness that is associated with increased numbers of airway mast cells. This model of viral bronchiolitis may be useful in experimental studies aimed at dissecting the pathogenetic role of airway mast cells in airway hyperresponsiveness after viral infection.

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