Temporal correlation of measurements of airway hyperresponsiveness in ovalbumin-sensitized mice

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Temporal correlation of measurements of airway hyperresponsiveness in ovalbumin-sensitized mice. Am J Physiol Lung Cell Mol Physiol 283: L219–L233, 2002. First published March 22, 2002; 10.1152/ajplung.00324.2001.—Airway hyperresponsiveness, airway inflammation, and reversible airway obstruction are physiological hallmarks of asthma. These responses are increasingly being studied in murine models of antigen exposure and challenge, using whole body plethysmography to noninvasively assess airway hyperresponsiveness. This approach infrequently has been correlated with indexes of airway hyperresponsiveness measured by invasive means. Furthermore, correlation with quantitative histological data for tissue infiltration by inflammatory and immune cells, particularly in the wall of airways, during daily airway challenge is lacking. To address these uncertainties, we used C57BL/6 mice that were immunized with ovalbumin or vehicle (saline) and sensitized to aerosolized ovalbumin or vehicle 8 days later. The mice were subsequently exposed to aerosolized ovalbumin or vehicle, respectively, on days 14–22. We assessed airway hyperresponsiveness to methacholine noninvasively on days 14, 15, 18, or 22; we studied the same mice 24 h later while they were anesthetized for invasive analyses of airway hyperresponsiveness. Plasma total IgE concentration was significantly higher in the ovalbumin-treated mice compared with the vehicle-treated mice, but this did not correlate with eosinophil number. Peak airway hyperresponsiveness measured by either approach correlated early during daily antigen challenge (days 14 and 15), but this correlation was lost later during subsequent daily antigen challenges (days 18 and 22). On days 14 and 15, peak airway hyperresponsiveness correlated with transmigration of neutrophils and macrophages, but not lymphocytes, in the peribronchovascular connective tissue sheaths. This extravascular accumulation was found to be focal by three-dimensional microscopy. We conclude that, although ovalbumin treatment changed lung function in mice, correlation between noninvasive and invasive measures of peak airway hyperresponsiveness was inconsistent.

Allergic asthma; murine model of asthma; pulmonary resistance; pulmonary dynamic compliance; inspiratory and expiratory times; whole body plethysmography; lung histopathology; quantitative histology; image analysis

AIRWAY HYPERRESPONSIVENESS, airway inflammation, and reversible airway obstruction are physiological hallmarks of asthma (22), yet the mechanisms that govern these pathophysiological responses are not fully understood. These hallmarks of asthma are being examined in murine models of allergic asthma, where manipulation can be applied to identify components of the underlying responses (18, 20, 21, 25). The marriage between the genetically manipulatable murine system and induced airway hyperresponsiveness has defined roles for inflammatory and immune regulatory molecules, but fundamental issues remain unresolved. These include the requirement and contribution for influx of specific immune-effector cell subtypes (7, 9, 15, 21), the basis for different responses among mouse strains (21), and the contributions of route and sequence of antigen exposure (9, 24).

Increasingly, murine models of antigen exposure and challenge are being evaluated physiologically, using a recording system (whole body plethysmography) that noninvasively assesses airway hyperresponsiveness to methacholine (4, 5, 8, 14). This noninvasive physiological approach offers the advantages of eliminating the effects of anesthesia and surgical trauma and permitting repeated assessment of the same mice while they breathe spontaneously. The noninvasive index of airway hyperresponsiveness, enhanced pause (P_enh), is an empirically derived, unitless value based on the pressure waveform in the plethysmograph box (8). However, the physiological meaning of P_enh compared with conventional methods of measuring lung resistance (R_l) and compliance (16) has been incompletely investigated (8, 17). One question that has not been addressed is whether P_enh reliably detects changes in airway hyperresponsiveness as the number of days of allergen exposure is increased. Furthermore, there has been no correlation of airway hyperresponsiveness with quantitative histological analysis of tissue infiltration of inflammatory and immune cells, particularly in the walls of airways, at various times during allergen exposure.

In the present study, we used a murine model of airway hyperresponsiveness (3) to ask two questions. First, we asked whether the noninvasive method for...
assessing changes in airway hyperresponsiveness in mice is reliable during repeated exposure to aerosolized allergen. To this end, we immunized and sensitized C57BL/6 mice to ovalbumin before exposing them daily to aerosolized ovalbumin and noninvasively assessing airway hyperresponsiveness to methacholine while the mice were awake and unrestrained. Twenty-four hours later, we measured $R_L$ and compliance invasively in the same mice while they were anesthetized. Control mice were treated with saline, the vehicle for ovalbumin. The second question we asked was if peak airway responsiveness correlated with transmigration of specific inflammatory and immune cells in the walls of airways. To address this question, we assessed airway hyperreactivity to methacholine while the mice were awake and unrestrained. Four hours later, we fixed the lung of these mice to identify and quantify leukocyte transmigration in the peribronchovascular connective tissue sheaths. We found that, while ovalbumin immunization and sensitization changed all of the airway parameters that we measured, correlation was inconsistent between a commonly employed noninvasive method and a widely accepted invasive method of assessing pulmonary mechanics. We also found that neutrophils and macrophages transmigrated into the peribronchovascular connective tissue sheaths in the allergen-treated mice, and this correlated well with changes in $R_L$.

**MATERIALS AND METHODS**

**Animals.** Pathogen-free, adult male C57BL/6 mice, 30–35 g body wt, were purchased from B&L Universal (Fremont, CA). Upon delivery, the mice were kept in a pathogen-free rodent facility and were provided food and water ad libitum. The animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Utah.

**Ovalbumin immunization and airway challenge.** Mice were immunized, on day 0, by intraperitoneal injection of 100 μg of ovalbumin (Sigma Chemical, St. Louis, MO) adsorbed to 1 mg of alum (Sigma Chemical) in a total volume of 0.5 ml of sterile PBS (Sigma Chemical). Later (8 days), we sensitized the airways to ovalbumin by exposing the mice to aerosolized 2% ovalbumin in sterile PBS for 30 min in a chamber (dimensions: 38 × 20 × 20 cm). This immunization and sensitization regimen emulated that used by Brusselle and colleagues (3). The efficacy of this immunization/sensitization protocol is shown in Plasma IgE concentration. Aerosolization was done using a DeVilbiss nebulizer (model 99; UltraNeb, Somerset, PA) driven by compressed air. Output of the nebulizer was 1 ml/min, with a mean particle diameter of 3.5 μm (manufacturer’s specification). On day 14 and daily thereafter for 8 days (days 15–22), the ovalbumin-immunized and -sensitized mice were exposed to aerosolized 2% ovalbumin in sterile PBS for 30 min.

We initially used the bias flow supply and its tubing to deliver the nebulized ovalbumin to the mouse plethysmograph chambers. However, this delivery route clogged the flow valves in the bias flow supply, particularly when nebulized albumin was used. This challenge was circumvented by delivering the nebulized ovalbumin in a separate exposure chamber, which enabled us to expose all the mice to the same concentration and duration of ovalbumin. The mice were then transferred to the plethysmograph chambers. These mice are designated “ovalbumin treated.”

Control mice were given an intraperitoneal injection of 0.5 ml of sterile PBS with 1 mg of alum, the vehicle for ovalbumin, on day 0. On days 8 and 14 and daily thereafter for 8 days (days 15–22), the control mice were exposed to aerosolized sterile PBS for 30 min in a chamber, as described above. These mice are designated “vehicle treated.”

**Respiratory system responses to methacholine provocation.** Respiratory system variables were assessed by two sequential approaches. The first approach used awake, unrestrained mice that were placed in a Plexiglas whole body barometric plethysmograph (Buxco Electronics, Sharon, CT; see Ref. 8). The same mice were then restudied 24 h later. At that time, the mice were anesthetized, a tracheostomy was made, their trachea was cannulated, their pleural spaces were opened, and the mice were placed in a rodent body box (16). The interval between each day’s nebulization of ovalbumin or saline and measurement of lung function was 4–6 h.

The Buxco system is composed of sealable, clear, cylindrical Lucite chambers (9 cm × 9 cm in length and width and 6 cm in height; ~500 cm³ in volume) connected to a gas flow and pressure control unit. Each Lucite chamber held one mouse. Four chambers were connected in parallel to the control unit so that simultaneous measurements were made for two ovalbumin-treated mice and two vehicle-treated (control) mice. The Buxco whole body barometric plethysmograph measured pressure changes within each chamber continuously as room air flowed through the chambers. Continuous recordings of $P_{	ext{awh}}$ were gathered on-line. This is a calculated parameter of airway obstruction (8). Among other respiratory system parameters that are measured are inspiratory time, expiratory time, and respiratory frequency.

Respiratory system variables were measured before (baseline) and after 10 min of methacholine nebulization (15 and 30 mg/kg). Measurements were recorded continuously for 15 min. Results are reported at the time when $P_{	ext{awh}}$ was at its peak. The time to peak $P_{	ext{awh}}$ response among the mice ranged between 3 and 8 min after nebulization was stopped, regardless of the two dosages of methacholine. Nebulized methacholine was delivered to a distribution reservoir (made by the manufacturer at our request), from which parallel hoses of identical length and diameter delivered the nebulized methacholine to four Lucite chambers simultaneously. The distribution reservoir ensured that all of the mice received the same concentration and duration of methacholine. The 10-min nebulization interval was chosen after a time-response curve was established (2, 5, and 10 min of nebulization). The two doses of methacholine were chosen after a dose-response curve was established (5, 10, 15, 30, and 50 mg/ml methacholine in sterile PBS). We selected two dosages of methacholine for the vehicle-treated (control) mice that, at the lower dosage, did not elicit airway hyperreactivity and at the higher dosage elicited minimal airway hyperreactivity. Reproducible, dose-dependent elevation of $P_{	ext{awh}}$ occurred with 15 and 30 mg/ml of methacholine in the ovalbumin-treated mice. Thus the two doses failed to induce changes in airway function by themselves but elicited airway hyperreactivity in the immunized and sensitized mice. The mice were allowed to recover for 45 min between the two doses of methacholine. Recovery was verified by the respiratory system variables returning to each mouse’s baseline values.

We also determined that it was critical to monitor and alter parameters for the rejected breath algorithm, without which the default algorithm rejected many breaths, thereby causing the tracings of respiratory system parameters for the ovalbumin-treated mice to appear to be shorter than control mice. This quality control issue was resolved with assistance from the manufacturer. Resolution involved lowering the
default threshold for rejecting breaths until the length of the tracing was equivalent for the two groups of mice. This is a selectable option in the software and thus is easy to change. Once the threshold for rejected breaths was established, the threshold was saved and used for the entire study.

Respiratory system variables were recorded at the following intervals 4–6 h after daily nebulization of ovalbumin (or vehicle) on day 14 (4–6 h), day 15 (24 h), day 18 (4 days), and day 22 (8 days). The experimental design is shown in Fig. 1. The same times were used to ascertain recruitment of leukocytes to the lung (see Leukocyte accumulation in lung tissue).

The assessment of airway physiology was then repeated in the same mice 24 h later to correlate $R_{m}$ to $R_{t}$ and dynamic compliance ($C_{dyn}$) measured directly. $R_{t}$ and $C_{dyn}$ were measured by a standard and widely accepted method (16). Briefly, the mice were treated with nebulized ovalbumin or saline as described for the noninvasive measurements. Later (4 h), the mice were anesthetized with pentobarbital sodium (60–70 mg/kg ip). An anterior, midcervical skin incision was made to expose the trachea, which was cannulated with an 18-gauge blunt needle. An opening was made in each side of the chest wall so that pleural pressure ($P_{pl}$) equaled body surface pressure. The tracheostomy catheter was passed through a hole in a plethysmograph box (clear Plexiglas cylinder 3.5 cm ID; 11.5 cm long; 0.35 cm wall thickness; 110.6 ml volume). One end (head end) of the cylinder was made of a Plexiglas plate, machined for an air-tight fit with an O-ring seal. The center of the end plate was perforated by an 18-gauge blunt needle, to which the tracheal cannula was attached. A tray was bonded to the end plate so that the mouse’s face could be placed against the end plate without disturbing the tracheal catheter’s connection. Mice were ventilated (Mouse Ventilator, model 687; Harvard Apparatus, Holliston, MA) initially with a tidal volume ($V_{t}$) of 5–6 ml/kg at 120 breaths/min and 3 cmH$_2$O of positive end-expiratory pressure. The dead space of the system was 0.03 ml. The mice were paralyzed with pancuronium bromide (0.3 mg/kg ip) to prevent respiratory movements. We used a 1-liter glass bottle filled with copper sponge to ensure isothermal conditions during measurements. Two small ports in the mouse-compartment end of the cylinder were used to monitor box pressure (very low range differential transducer, model DP45; Validyne Engineering, Northridge, CA). The reference side of that transducer was connected through a three-way stopcock to a second 1-liter glass bottle, also filled with copper sponge, to prevent atmospheric pressure swings. Changes in the box pressure provided volume ($V$) information. Airflow ($\dot{V}$) was derived from differentiation of the volume signal. The delay between the volume and flow signals was <0.5 ms. Transpulmonary pressure ($P_{tp}$) was measured with a second pressure transducer as the difference between airway pressure ($P_{aw}$), taken from the opening of the tracheal cannula, and the box pressure, which was equal to $P_{tp} = P_{aw} - P_{pl}$.

Measurements of $R_{t}$ and $C_{dyn}$ were made before (baseline) and after 30 tidal breaths of 15 and 30 ml/ml nebulized methacholine, delivered through the ventilator at 60 breaths/min. Forty-five minutes were allowed between the two doses of methacholine for the mice to recover to their baseline values. Peak responses to either dose were reached between 1 and 2 min of delivery of methacholine, regardless of the two dosages of methacholine. We report peak results. We analyzed the tracings of $V$, $\dot{V}$, and $P_{tp}$ at a respiratory rate of 120 breaths/min. $R_{t}$ was calculated as an average value for inspiration (insp) and expiration (exp) at isovolumes in the midtidal breathing as follows

$$R_{t}(\text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}) = \frac{[P_{aw} - P_{pl}(\text{insp})] - [P_{aw} - P_{pl}(\text{exp})]}{\dot{V}(\text{insp}) + \dot{V}(\text{exp})}$$

Values of both $R_{t}$ and $C_{dyn}$ are reported as the average of five consecutive breaths.

Leukocyte accumulation in lung tissue. We also determined the distribution and number of leukocytes that accumulated in the lungs of ovalbumin-treated mice compared with vehicle-treated mice on days 14, 15, 18, and 22 (days 15–22). Noninvasive measurement of pulmonary mechanics was performed on days 14, 15, 18, and 22 (days 14–22). Invasive measurement of pulmonary mechanics was performed on the same mice 24 h later to correlate $R_{m}$ to $R_{t}$ and dynamic compliance ($C_{dyn}$) measured directly. Later (4 h), the mice were anesthetized with pentobarbital sodium (100 mg/kg ip). Their tracheas were cannulated, their rib cages were opened, and their lungs were inflated with air to ~75% total lung capacity. The hilum of the left lung was cross-clamped to maintain its inflation and to trap vascular contents in its blood vessels. The left lung was removed, with the clamp attached to its hilum, and immersed in 10% buffered neutral formalin at 4°C overnight. The next day, the left lung was processed and embedded whole in paraffin wax. The right lung was allowed to collapse and then infused with OCT compound (VWR, Media, PA). The right lung was immersed in OCT compound and frozen.

Three-dimensional stereomicroscopy was used to assess accumulation of leukocytes in the lung tissue. For this analysis, we cut 60- to 100-μm-thick slabs of lung tissue, stained them with hematoxylin and eosin (H&E), and observed the slabs with the aid of an Edge three-dimensional microscope (Edge Scientific Instrument, Santa Monica, CA) equipped

![Image](http://ajplung.physiology.org.org/attachment.php/attachment/ajplung/108/5968155.png)
with fluorescence illumination. Stereopair photographs were taken of the autofluorescent stains.

Immunohistochemistry was performed on paraffin-embedded sections (26). Briefly, tissue sections (4–5 μm) were collected on PLUS slides (VWR). The sections were treated with 3% H₂O₂ in methanol for 10 min at 37°C to remove endogenous peroxidase. The sections were washed with PBS, blocked with normal goat serum, and then incubated with purified rat anti-mouse monoclonal primary antibodies. The primary antibodies were directed against neutrophils (rat anti-mouse neutrophil antibody; Caltag Laboratories, Burlingame, CA; see Ref. 11), activated macrophages (rat anti-Mac-3 antibody; PharMingen, San Diego, CA; see Ref. 6), and T lymphocytes and some B lymphocytes (rat anti-CD5 antibody; PharMingen; see Ref. 13). Optimal dilutions were 1:200 for the rat anti-mouse neutrophil antibody, 1:1,000 for the anti-Mac-3 antibody, and 1:200 for the anti-CD5 antibody at 4°C overnight. Staining controls included omission of the primary antibody, omission of the secondary antibody (biotinylated IgG), and substitution of the primary antibody with species-matched, isotype-matched irrelevant antibody (insulin). Furthermore, we immunostained smears of mouse peripheral blood to confirm cell-specific labeling. For the rat anti-mouse neutrophil and CD5 primary antibodies, antigen detection was done by the tyramide signal amplification method (NEL 700 kit; NEN Research Products, Boston, MA). For the rat anti-Mac3 primary antibody, antigen detection was done by the avidin-biotin-peroxidase method (ABC Elite kit; Vector Laboratories, Burlingame, CA). Both antigen detection methods were used for the anti-insulin antibody. We used Gill’s no. 3 hematoxylin to counterstain the immunostained tissue sections. Photography was done with the aid of a Zeiss Axioshot light microscope. We expressed the results as the number of extravascular leukocytes for each leukocyte type per millimeter of airway basal lamina length, which was measured by tracing the basal lamina in calibrated digital images (Bioquant True Color Image Analysis System; R & M Biometrics, Nashville, TN). We also traced the outside perimeter of the peribronchovascular connective tissue sheaths. The subtended area surrounding each bronchiolar was the extravascular tissue space in which leukocytes were counted by electronic touch count. This quantitative morphological analysis of extravascular leukocytes in the peribronchovascular connective tissue sheaths was performed on one tissue section that was cut from each mouse’s entire left lung on days 15, 18, and 22. The number of peribronchovascular connective tissue sheaths that were analyzed per left lung ranged between 15 and 20. We did not perform this analysis on lung tissue sections cut from the mice that were killed on day 14 because leukocytes had insufficient time to transmigrate in the peribronchovascular connective tissue sheaths 4 h after nebulization of ovalbumin, which is the time when these mice were killed.

Bronchoalveolar lavage cell counts. Replicate experiments, including whole body plethysmography using the Buxco system, were performed using ovalbumin-treated and vehicle-treated mice. After methacholine challenge and measurement of airway hyperresponsiveness, the mice were anesthetized with pentobarbital sodium (60–70 mg/kg ip). Their tracheas were cannulated, and their chests were opened. Bronchoalveolar lavage (BAL) was performed five times (0.8 ml PBS/lavage) through the tracheal cannula. The retrieved lavage aliquots were pooled and centrifuged, from which the cell pellet was resuspended in PBS and counted using a hemocytometer. Slide smears were treated with Wright’s stain (Sigma Chemical) for differential cell counts.

**Plasma IgE concentration.** The ovalbumin-treated and vehicle-treated mice that were used to measure leukocytes in BAL fluid were also used to measure plasma total IgE concentration. Blood was withdrawn to obtain the plasma layer, which was analyzed by enzyme-linked immunosorbent assay for plasma IgE concentration, using a rat anti-mouse IgE monoclonal antibody (clone R35–7; PharMingen) and the manufacturer’s protocol. Measurements were made in triplicate, the average for which is reported.

**Statistical analysis.** The results are shown as means ± SD (n = 4–10 mice/group, as described in RESULTS and legends for Figs. 1–11). Unpaired t-test was used to detect differences between the ovalbumin-treated and vehicle-treated mice (23). Simple linear regression and correlation tests were used to identify relationships among airway responsiveness parameters and between leukocyte subtypes in the walls of airways and airway responsiveness parameters (23). Fisher’s r-to-z test was used to identify statistically significant correlations (23). We used StatView 5.0 (Abacus Concepts, Berkeley, CA) and accepted P < 0.05 as indicating statistical significance.

**RESULTS**

**Plasma IgE concentration.** Plasma total IgE concentration transiently increased in the ovalbumin-immunized and -sensitized mice. For example, plasma total IgE concentration was 3.0 ± 1.3 μg/ml (mean ± SD; n = 4) on day 15, 21.3 ± 3.7 μg/ml on day 18 (n = 4), and 4.7 ± 7.2 μg/ml (n = 4) on day 22. The concentration of total plasma IgE was statistically different on day 18 compared with days 15 and 22 (P < 0.05). IgE was not detected in the plasma of the vehicle-treated mice.

Peak P enh and R L are increased, whereas C dyn and the ratio of inspiratory to expiratory time are decreased, in ovalbumin-treated mice. P enh is commonly used to investigate alterations in airway responsiveness in awake, unrestrained mice (8), but it is an empirical parameter that may reflect changes in the respiratory system in addition to changes in airway responsiveness. For this reason, we assessed P enh and the ratio of inspiratory time to expiratory time (T i/T e), both analyzed by the noninvasive method, and compared the results with directly measured parameters of airway responsiveness (R L and C dyn). The latter two measurements were made 24 h after the noninvasive measurements (see MATERIALS AND METHODS and Fig. 1). The daily physiological results for ovalbumin-treated and vehicle-treated mice are summarized in Fig. 2.

Peak P enh and R L were significantly higher in the ovalbumin-treated mice compared with the matched vehicle-treated mice, regardless of the dose of methacholine or day of study (Fig. 2). The only exception occurred on day 18 for R L at 30 mg/ml methacholine, because of variability among the ovalbumin-treated mice. In general, the P enh and R L results were higher during airway provocation with 30 mg/ml methacholine compared with 15 mg/ml methacholine. These differences are expected physiological indicators of airway obstruction.
When Penh was at its peak, peak Cdyn and the Tᵢ/Tₑ ratio were lower in the ovalbumin-treated mice compared with the matched vehicle-treated mice, regardless of the dose of methacholine or day of study (Fig. 2). The only exceptions occurred on day 22 for Cdyn at both doses of methacholine and on day 22 for the Tᵢ/Tₑ ratio at 30 mg/ml methacholine, again because of variability among the mice. The Tᵢ/Tₑ ratio was lower because the inspiratory time was decreased, whereas the expiratory time was increased (data not shown). These differences also are expected physiological indicators of airway obstruction.

Peak Penh inconsistently correlated with Rₐ, Cdyn, and the Tᵢ/Tₑ ratio in ovalbumin-treated mice. The first question that our study addressed was whether Penh reliably identified changes in airway hyperresponsiveness during eight consecutive days of allergen exposure and methacholine provocation. Early in the course of daily exposure to nebulized ovalbumin (days 14 and 15, regardless of methacholine dose), peak Penh correlated with peak Rₐ, Cdyn (Fig. 4), and the Tᵢ/Tₑ ratio (Fig. 5). Later in the course of daily exposure to nebulized ovalbumin (days 18 and 22), however, peak Penh did not correlate well with peak Rₐ, Cdyn, or the Tᵢ/Tₑ ratio (Figs. 3–5).

Leukocytes accumulated in the walls of arteries, airways, and veins in ovalbumin-treated mice. We also sought to correlate the changes in airway hyperresponsiveness after exposure to nebulized ovalbumin with...
the accumulation of inflammatory and immune cells in the peribronchovascular connective tissue sheaths. We first examined Wright-stained sections of lung tissue. This analysis revealed that neutrophils and mononuclear cells accumulated in the walls and surrounding interstitium of intrapulmonary arteries and airways (Fig. 6) and veins (Fig. 7) in the lungs of the ovalbumin-treated mice compared with the vehicle-treated mice. Particularly notable was the margination and transmigration of leukocytes in as little as 4 h (day 14) after exposure to nebulized ovalbumin. Accumulation increased over the succeeding 24 h. The inflammatory infiltrates remained in the walls and surrounding interstitium of arteries, airways, and veins throughout the 8 days of ovalbumin exposure (Figs. 6 and 7).

We obtained a broader perspective on the tissue distribution of the transmigrated cells by examining the autofluorescence of H&E-stained thick sections (60 µm) of lung, using three-dimensional fluorescence microscopy that allows imaging of thick sections to provide a depth perspective. This technique showed focal aggregation inside and outside pulmonary arteries (Fig. 8; stereopair) and in the wall of the neighboring airways (Fig. 8) and veins (data not shown). Imaging the vascular and airway structures in this fashion resulted in a novel observation. Margination of leuko-

*Fig. 3. Regression plots comparing peak P_{enh} (independent parameter) and R_{L} (dependent parameter) in response to 2 doses of nebulized methacholine (15 and 30 mg/ml) on days 14, 15, 18, and 22 in ovalbumin-treated mice (○) and vehicle-treated mice (○). P_{enh} was assessed noninvasively, using whole body plethysmography. R_{L} was assessed invasively 24 h later, using a rodent body box. When P_{enh} was at its peak, both P_{enh} and R_{L} were consistently greater in the ovalbumin-treated mice compared with the vehicle-treated mice on the same day. Correlation coefficients ("C" in each graph) were significant (*P < 0.05 by Fisher's r-to-z test) on days 14 and 15 in response to 15 mg/ml methacholine and on days 14, 15, and 18 in response to 30 mg/ml methacholine. The circles represent results for individual mice. Some open circles (vehicle-treated mice) overlap.
cytes in and transmigration across pulmonary arteries occurred around the half of the pulmonary arteries adjacent to the neighboring airway.

**Neutrophils and macrophages comprised the tissue infiltrates in ovalbumin-treated mice.** We used immunohistochemistry as the second step to test for correlation between changes in airway hyperresponsiveness after exposure to nebulized ovalbumin with an inflammatory and immune cell infiltrate. Immunohistochemistry was used to differentiate among the leukocytes that accumulated in the peribronchovascular connective tissue sheaths surrounding intrapulmonary airways and arteries, according to the expression of surface antigens specific for neutrophils (neutrophil antibody positive), activated macrophages (Mac3 antibody positive), and lymphocytes (CD5 antibody positive; T lymphocytes and some B lymphocytes). Representative micrographs of the immunopositive cells in the ovalbumin-treated and vehicle-treated mice are shown in Fig. 9. Quantitative histology was then used to estimate the number of leukocytes per millimeter of airway basal lamina for both groups of mice (Fig. 10). We focused on the leukocytes that accumulated in the peribronchovascular connective tissue sheaths because inflammatory mediators released from those infiltrating leukocytes may affect airway smooth muscle reactivity. Among the ovalbumin-treated mice, neutrophils and activated macrophages were the first and...
predominant types of leukocyte that accumulated in the peribronchovascular connective tissue sheaths. Neutrophil infiltration increased from day 15 to day 18 and then diminished on day 22 (Fig. 10). Activated macrophage infiltration increased from day 15 to day 22 (Fig. 10). CD5-positive lymphocytes also infiltrated the walls of airways of the ovalbumin-treated mice. However, accumulation of CD5-positive lymphocytes was modest compared with neutrophils and activated macrophages (Fig. 10).

Eosinophils, identified by characteristic staining of their granules in the H&E-stained sections, were seen in the peribronchovascular connective tissue sheaths of the ovalbumin-treated mice. However, they were seen the least among the infiltrating leukocytes. The small number of tissue eosinophils in the ovalbumin-treated mice occurred despite a robust elevation in plasma total IgE concentration, as described above.

Neutrophil and macrophage accumulation around airways is correlated to lung function variables in ovalbumin-treated mice. We used the quantitative immunohistochemical results to test for correlation between airway hyperresponsiveness and inflammatory cell accumulation in the peribronchovascular connective tissue sheaths after exposure to nebulized ovalbumin (Table 1). Neutrophil and activated macrophage accumulation correlated with $P_{meh}$, $R_L$, and $C_{dyn}$ on days 15, 18, and 22 (Table 1). Lymphocyte accumulation, however, did not correlate well or consistently

Fig. 5. Regression plots comparing peak $P_{meh}$ (independent parameter) and the $T/T_e$ ratio (dependent parameter) in response to 2 doses of nebulized methacholine (15 and 30 mg/ml) on days 14, 15, 18, and 22 in ovalbumin-treated mice (●) and vehicle-treated mice (○). $P_{meh}$ and the $T/T_e$ ratio were assessed noninvasively, using whole body plethysmography. When $P_{meh}$ was at its peak, $P_{meh}$ was consistently greater, and the $T/T_e$ ratio was consistently lower in the ovalbumin-treated mice compared with the vehicle-treated mice on the same day. Correlation coefficients were significant (*) by Fisher’s r-to-z test) on days 14 and 15 in response to 15 mg/ml methacholine and on day 14 in response to 30 mg/ml methacholine. The circles represent results for individual mice. Some open circles (vehicle-treated mice) overlap.
with $P_{\text{enh}}$, $R_L$, and $C_{\text{dyn}}$ (Table 1). Eosinophil accumulation was insufficient to analyze statistically.

Increased BAL cell counts in ovalbumin-treated mice did not reflect interstitial infiltrates early in the time course. We found that BAL fluid from the ovalbumin-treated mice had more leukocytes per microliter than the vehicle-treated mice. On day 15, lavage leukocyte number was $2.4 \pm 1.5$ vs. $3.5 \pm 3.3/\mu\text{l}$ for the ovalbumin-treated and vehicle-treated mice, respectively (not significant). On day 18, the number of leukocytes in lavage fluid was $39.1 \pm 23.8$ vs. $5.4 \pm 1.4/\mu\text{l}$ for the ovalbumin-treated and vehicle-treated mice, respectively ($P < 0.05$). On day 22, lavage leukocyte number was $11.1 \pm 5.3$ vs. $3.9 \pm 0.4/\mu\text{l}$ for the ovalbumin-treated and vehicle-treated mice, respectively ($P < 0.05$). The lavage fluid contained more neutrophils, alveolar macrophages, and lymphocytes retrieved from the ovalbumin-treated mice than the vehicle-treated mice (Fig. 11). Neutrophils appeared first in the lavage fluid (day 15; $P < 0.05$). Variability was such, however, that few statistically significant differences were detected, despite having seven mice per group per day. Eosinophils were infrequently observed in the BAL fluid, so their number was too small to analyze statistically.

The ratio of tissue leukocytes (Fig. 9) to lavage leukocytes (Fig. 11) for neutrophils, macrophages, or lymphocytes in the ovalbumin-treated mice was skewed early in the course in favor of more tissue leukocytes than lavage leukocytes (Table 2). This disproportionate distribution of leukocytes early in the course indicates that the abundance of leuko-
cytes in lavage fluid underestimated leukocyte abundance in tissue.

DISCUSSION

The purposes of our study were to use a murine model of allergen-induced airway inflammation (3) to test the reliability of a noninvasive method for assessing changes in airway hyperresponsiveness and to identify whether early changes in airway hyperresponsiveness are related to pulmonary infiltration of inflammatory and immune cells. The noninvasive method that we used for assessing changes in airway responsiveness was barometric whole body plethysmography, using the empirical and unitless parameter Penh and the T1/Tc ratio. An invasive method for measuring airway responsiveness (RL and CDyn) was used.

Fig. 7. Lung histology demonstrating the inflammatory responses in the perivascular connective tissue sheaths surrounding intrapulmonary veins (PV) of ovalbumin-treated mice (a–c; n = 6–7 mice) and vehicle-treated mice (d–f; n = 6–7 mice) on days 14, 15, and 22. On each day, the lungs were fixed for histology 4 h after ovalbumin nebulization and noninvasive assessment of pulmonary mechanics. The tissue sections are stained with hematoxylin and eosin. For the ovalbumin-treated mice, leukocytes (arrows) are marginated in the lumen of intrapulmonary veins early (day 14) and in the walls of intrapulmonary veins later (days 15, 18, and 22). Histology on day 18 is not shown because it was the same as on day 22. a–f are the same magnification (scale bar is 20 μm).

Fig. 8. Stereo pair micrographs of lung tissue from a mouse on day 15 of ovalbumin exposure. Thick tissue sections (60 μm) were stained with hematoxylin and eosin and observed with a 3-dimensional microscope equipped with fluorescence illumination. Yellow autofluorescence is emitted from cytoplasm (eosin stain), whereas red autofluorescence is emitted from nuclei (hematoxylin stain). The aggregates of red dots (arrows) in the lumen of the intrapulmonary artery and between it and the neighboring airway are infiltrates of leukocytes. Note that the leukocyte infiltrate is focal and that it is located on the half of the intrapulmonary artery that faces its neighboring airway. Original magnification was ×10. The airway is 1 mm in diameter.
to isolate airway function from other respiratory system influences in the same mice. Peak $P_{\text{enh}}$ and $R_L$ were increased, whereas the $T_i/T_e$ ratio and $C_{\text{dyn}}$ were decreased, in the ovalbumin-treated mice compared with the vehicle-treated mice. Peak $P_{\text{enh}}$ correlated with peak $R_L$, the $T_i/T_e$ ratio, and $C_{\text{dyn}}$ in the first 2 days only (days 14 and 15). Importantly, these correlations were lost at later times (days 18 and 22) and were lost for unknown reasons. We conclude that the noninvasive method for assessing changes in airway hyperresponsiveness in ovalbumin-treated mice provides an inconsistent indication of airway hyperresponsiveness to methacholine.

Coincident with the rapid airway functional changes were rapid margination and transmigration of inflammatory and immune cells in the peribronchovascular connective tissue sheaths. Accumulation of neutrophils and activated macrophages around airways correlated well with peak $P_{\text{enh}}$, $R_L$, and $C_{\text{dyn}}$ early (day 15), but the correlations disappeared at later times (days 18 and 22). Accumulation of lymphocytes correlated poorly with peak $P_{\text{enh}}$, $R_L$, and $C_{\text{dyn}}$, regardless of the day of study. Eosinophil accumulation in the tissue was insufficient to test for correlation with indexes of airway hyperresponsiveness. Finally, leukocyte number and differential percentage in BAL fluid did not accurately
reflect the temporal accumulation of inflammatory and immune cells in lung tissue sections. We conclude that airway hyperresponsiveness in mice exposed to ovalbumin is related, in part, to accumulation of neutrophils and activated macrophages in the wall of airways.

Eosinophils in BAL fluid is typically a hallmark of asthma models in immunologically intact mice (2, 3, 7, 8, 14, 25). Our study showed that eosinophils accumulated in the lung but that their accumulation was less than neutrophils, activated macrophages, and CD5-positive lymphocytes. Minimal accumulation of eosinophils in lung tissue or BAL in allergen-induced lung inflammation in mice is not a new observation, however. Several studies have shown a paucity of eosinophils in either lung tissue or BAL fluid of allergen-exposed mice (2, 4, 10, 21). Our results provide another example of airway hyperreactivity in mice that occurs without eosinophils.

Why our mouse model of ovalbumin-induced airway inflammation and hyperresponsiveness failed to elicit large numbers of eosinophils remains unclear. This observation was surprising, given the robust increase in total plasma IgE concentration that occurred in the ovalbumin-treated mice. One possible explanation is our protocol used only one intraperitoneal injection of ovalbumin, whereas studies that detected increased numbers of eosinophils in blood and BAL used two or

![Image](https://www.ajplung.org/)

**Table 1. Correlation between airway variables and leukocyte infiltration in the walls of airways**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Neutrophils</th>
<th>Mac3-Positive Macrophages</th>
<th>CD5-Positive Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 15</td>
<td>Day 18</td>
<td>Day 22</td>
</tr>
<tr>
<td>Pemh</td>
<td>0.750*</td>
<td>0.336</td>
<td>0.809*</td>
</tr>
<tr>
<td>RL</td>
<td>0.823*</td>
<td>0.887*</td>
<td>0.545</td>
</tr>
<tr>
<td>Cdyn</td>
<td>−0.851*</td>
<td>−0.872*</td>
<td>−0.735*</td>
</tr>
<tr>
<td>T/Ti</td>
<td>−0.382</td>
<td>−0.641</td>
<td>−0.656</td>
</tr>
</tbody>
</table>

RL, resistance of the lung; Cdyn, dynamic compliance of the lung; T/Ti, inspiratory time-to-expiratory time ratio. *P < 0.05 by simple linear correlation and Fisher’s r-to-z comparison.

**Fig. 10.** Summary histogram showing accumulation of leukocyte subtypes in the peribronchovascular connective tissue sheaths surrounding bronchioles on days 15, 18, and 22 in ovalbumin-treated mice (solid bars; n = 7/day) and vehicle-treated mice (open bars; n = 7/day). Data are means ± SD. Quantitative histology was used to estimate the number of neutrophils (PMN), Mac3-positive macrophages, and CD5-positive lymphocytes/mm airway basal lamina. The three types of leukocytes were identified immunohistochemically (see Fig. 8). The number of each type of leukocyte/mm distal airway basal lamina was determined by image analysis. The number of immunopositive cells/type of leukocyte in the peribronchovascular connective tissue sheaths surrounding each bronchiole was divided by the length of that airway’s basal lamina, measured by calibrated tracing. Neutrophil and Mac3-positive macrophage accumulation was statistically greater in the ovalbumin-treated mice compared with the vehicle-treated mice on days 15 and 18 (*P < 0.05 by unpaired t-test). On day 22, neutrophil accumulation decreased in the ovalbumin-treated mice to a level that was equivalent to that in the vehicle-treated mice. On the same day, Mac3-positive macrophage accumulation increased further in the ovalbumin-treated mice compared with the vehicle-treated mice. CD5-positive lymphocytes (T and some B lymphocytes) were equivalent between ovalbumin-treated and vehicle-treated mice. Only on day 22 was accumulation of CD5-positive lymphocytes statistically greater in the ovalbumin-treated mice compared with the vehicle-treated mice.

**Fig. 11.** Summary histogram showing retrieval of leukocyte subtypes by bronchoalveolar lavage (BAL) on days 15, 18, and 22 in ovalbumin-treated mice (filled bars; n = 4/day) and vehicle-treated mice (open bars; n = 4/day). Data are means ± SD. The 3 types of leukocytes were identified by cytological characteristics in stained slide smears (see Fig. 8). Neutrophil, macrophage, and lymphocyte accumulation peaked on day 18. Because of variability, however, few statistical differences were detected between the ovalbumin-treated mice and vehicle-treated mice. The number of neutrophils retrieved from ovalbumin-treated mice was significantly greater compared with vehicle-treated mice on days 15 and 22 (*P < 0.05 by unpaired t-test). No differences were detected statistically for macrophages between the two groups of mice, regardless of day of study. The number of lymphocytes retrieved from ovalbumin-treated mice was significantly greater compared with vehicle-treated mice on day 22 only (*P < 0.05 by unpaired t-test).
more intraperitoneal (or subcutaneous) injections of ovalbumin before aerosol sensitization (8, 20, 25). On the other hand, Brusselle and colleagues (3) used a single intraperitoneal injection of ovalbumin, followed by repeated exposure to aerosolized ovalbumin, and observed accumulation of eosinophils. We followed their protocol. Another possibility is the strain of mouse appears to be a determinant of eosinophil recruitment and airway hyperresponsiveness (4, 5, 21, 25). In this regard, we and Brusselle and coworkers (3) used C57BL/6 mice.

We compared two standard approaches to measure lung function in the same mice. The first, and commonly used today, is Penh. The second is to directly measure $R_L$ and $C_{dyn}$. The first approach is noninvasive and readily determined, whereas the second approach is invasive and technically more challenging. We found that correlation between the two approaches was inconsistent, in that correlation occurred during the first days of daily aerosolization of ovalbumin, but correlation did not occur during later days of daily aerosolization. This outcome is not suspect because Penh does not directly measure the function assessed by the invasive approach (17). However, it is clear that such comparisons can be unreliable and that Penh cannot simply stand in for $R_L$. Until other noninvasive measurements are available, however, a reasonable design is to compare the noninvasive and invasive results for the same mice. Another group of investigators has found inconsistent results when Penh measurements are compared with lung mechanics measurements (19). Combined, their results and our results suggest that Penh is unreliable for characterizing lung mechanics.

Penh is an empiric parameter that changes as a consequence of bronchoconstriction (8). It is derived from analysis of the waveform of the plethysmograph box pressure and pressure in a reference chamber. Penh is calculated from the formula

$$\text{Penh} = \frac{\text{peak expiratory pressure}}{\text{peak inspiratory pressure}} \times \frac{\text{expiratory time} - \text{relaxation time}}{\text{relaxation time}}$$

as described by Hamelmann and associates (8). The unitless Penh parameter reflects changes in the waveform of the box pressure from both peak inspiration (peak inspiratory pressure) and peak expiration (peak expiratory pressure) and combines the waveform with the timing comparison of early and late expiration (also called “pause”). Thus Penh is an arbitrary mathematical function of the proportion of the box pressure signal from inspiration and expiration and the timing of expiration. Although a number of studies, including the present study, have established that bronchoconstriction increases Penh, the opposite question of whether increased Penh is a consequence of changes in the mechanical properties of the respiratory system has not been established. Other potential contributors to the plethysmograph box pressure signal are differences in the temperature and humidity of inspired and expired air, as well as changes in respiratory rate, $V_t$, or phase lags between nasal and thoracic flow (compression; see Ref. 12). Hamelmann and colleagues (8) showed that these other potential contributors changed asynchronously with Penh and therefore concluded that Penh is a valid indicator of bronchoconstriction in mice. Our results and those recently reported by Peták and colleagues (19) suggest that increased Penh may not be a consequence of changes in the mechanical properties of the respiratory system because correlation is inconsistent between Penh and more widely accepted measurements of lung mechanics.

The regression plots (Figs. 3–5) for the ovalbumin-treated and vehicle-treated groups of mice were clustered later in the time course (days 18 and 22). This clustering was associated with poor correlation between peak Penh and direct measures of allergen-induced airway obstruction. This is a new observation. An explanation for this observation relates to experimental design. We measured airway responsiveness on 4 of 8 days of daily nebulization of ovalbumin. Other investigators who have used whole body plethysmography and direct measures of airway obstruction in mice evaluated changes in airway responsiveness just one time after two or three repetitions of antigen exposure (4, 5, 8). Therefore, the number of daily repetitions of antigen exposure appears to influence indexes of airway obstruction. Other explanations are possible for the difference between our results and those reported by other investigators. For example, no two studies, including ours, have used the same allergen exposure regimen. The regimens have used different types, sources, and concentrations of allergen; different routes of sensitization; different intervals of time between sensitization and airway challenge; different numbers of airway challenges and intervals between repeated challenges; and different concentrations of methacholine (4, 5, 8). Therefore, translating the results from one physiological study to another is difficult. Another explanation may be genetic background. Several studies have shown that BALB/c mice demonstrate greater airway responsiveness after aller-

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<tr>
<th>Day 15</th>
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<th>Day 22</th>
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<th>Day 15</th>
<th>Day 18</th>
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<tbody>
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<td>Neutrophils</td>
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<td>2/1</td>
<td>6/1</td>
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<td>Lymphocytes</td>
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Units for tissue leukocytes are cells/mm airway basal lamina perimeter. Units for air space leukocytes are cells/μl of lavage fluid.

Table 2. Ratio of tissue leukocytes and air space leukocytes in ovalbumin-treated mice

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Oxygen exposure than C57BL/6 mice (4, 21, 25). We used C57BL/6 mice because we are using this strain for the genetic background of transgenic and knockout mice. We have not evaluated other strains of mice.

A novel observation provided by our study is the focal nature of leukocyte margination in and transmigration across pulmonary arteries and veins (Fig. 8). This observation was made possible by the use of a three-dimensional microscope to evaluate thick sections (60–100 μm) of lung tissue. The advantage of this technique is that it enables depth to be appreciated in a single thick slab of tissue, whereas typically relatively thin tissue sections (5 μm) are used to evaluate histopathology (Figs. 6 and 7). Although observation of 5-μm-thick tissue sections has localized the site of leukocyte margination in and transmigration across pulmonary arteries and veins, in addition to alveolar capillaries, in the lungs of allergen-challenged mice (2–5, 21, 25) and in the lungs of sheep during air embolism-induced acute lung injury (1), appreciation that the sites of margination and transmigration are focal and may occur in locations besides capillaries has been lacking. An interesting subjective impression that derived from observing the tissue slabs three-dimensionally is that the focal spots of leukocyte margination in and transmigration across pulmonary arteries were on the half of the artery that faced the neighboring airway. Other investigators, using thin sections, have not seen this association (2). Our observation raises questions about the regulation of directed margination and transmigration in the lung when an inflammatory stimulus is delivered on the airway side of the air-blood barrier.

We also noted that accumulation of leukocytes in the peribronchovascular connective tissue sheaths (Fig. 10) and retrieval of leukocytes in BAL fluid (Fig. 11) did not match. Accumulation of leukocytes in lung tissue occurred sooner than revealed by retrieval of leukocytes in BAL fluid. Although this observation makes sense intuitively, the observation serves as a reminder that leukocyte counts in BAL fluid are temporally delayed compared with tissue inflammatory responses.

We conclude that ovalbumin sensitization alters airway reactivity in mice over at least an 8-day period during daily exposure to aerosolized ovalbumin. The empiric, unitless parameter (Penh) was statistically different between ovalbumin-treated and -untreated mice throughout the 8-day study period. However, peak Penh correlated with direct measurements of airway obstruction (Rl and Cdyn) only during the early days of the experimental protocol. At later days of analysis, peak Penh, Rl, and Cdyn varied independently. Thus Penh appears to have limitations as an index of airway obstruction.

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REFERENCES


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