The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration

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Early-phase reactions (EPRs) and late-phase reactions (LPRs) are characteristic features of bronchial asthma, although the pathogenetic mechanisms responsible for each of the responses are not fully defined. A murine model of EPRs and LPRs was developed to investigate the role of IL-5 and eosinophils in development of both responses. After initial intraperitoneal sensitization and airway challenge to ovalbumin (OVA), mice were provoked by additional exposure to OVA. An EPR, characterized by a transient increase in airway responsiveness, was observed 5–30 minutes after antigen provocation. This response was followed by an LPR that reached its maximum at 6 hours after challenge and was characterized by increased airway responsiveness and significant lung eosinophilia. The EPR was blocked by cromoglycate and albuterol, whereas the LPR was abolished by cromoglycate and hydrocortisone. Before provocation with allergen, administration of anti–IL-5 antibody prevented the influx of eosinophils into the lung tissue and abolished the LPR but not EPR. These results suggest that IL-5 and eosinophils are essential for development of the LPR, but not EPR, in this model.


Introduction

Allergen provocation of allergic asthmatics characteristically leads to reproducible patterns of bronchoconstrictive responses. Some subjects respond with an early asthmatic response, or early-phase reaction (EPR), with maximal airway narrowing occurring within 15–30 minutes and returning to baseline within 1–2 hours. Approximately 60% of subjects also develop a second, late asthmatic or late-phase, response that commences after 3–5 hours, is maximal at 6–12 hours, and may persist for up to 24 hours (1, 2). Mechanistically, these responses probably reflect different processes, as the EPR is blocked by nedocromil, albuterol, and cromoglycate, and the late-phase reaction (LPR) is abolished by nedocromil, cromoglycate, and steroids when given before allergen provocation (3–5). Although the EPR appears to depend largely on the release of mediators from airway mast cells, leading to bronchoconstriction and airway edema, the development of the LPR and the concomitant increases in airway reactivity are associated with an influx and activation of inflammatory cells, particularly lymphocytes and eosinophils in the bronchial mucosa (6–9).

To understand more fully the complex pathophysiological mechanisms underlying asthma and the accompanying changes in lung function, we have developed an animal model that mimics the asthmatic disease state. Murine models of allergic airway disease have been well defined in recent years; however, a distinction between the EPR and LPR has not been demonstrated (10–16). Introduction of whole-body barometric plethysmography in conscious, unrestrained animals enabled us to monitor changes in airway function in a longitudinal fashion, compared with the more traditional invasive systems in which airway changes can be measured only at single time points (17). Moreover, this approach was effective for monitoring several animals simultaneously, allowing us to define an EPR and LPR in sensitized and challenged mice. In these investigations, we have begun to approach the mechanistic aspects of both phases of the response. The pulmonary changes induced by allergen provocation and pharmacological characterization of the EPR and the LPR were very similar to observations in asthmatics. These studies clearly define the role of IL-5 and eosinophils in LPRs but not EPRs.

Methods

Animals. Female BALB/c mice, free of murine specific pathogens, were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). The mice were maintained on a diet free of ovalbumin (OVA). All experi-
mental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

**Experimental protocol.** Mice, 10–12 weeks of age, were sensitized on days 1 and 14 by intraperitoneal injection of 20 μg OVA (Grade V; Sigma Chemical Co., St. Louis, Missouri, USA) emulsified in 2.25 mg aluminum hydroxide (AlumImuject; Pierce Chemical Co., Rockford, Illinois, USA) in a total volume of 100 μL. Mice were challenged daily with OVA for 20 minutes via the airways (1% in saline) for 3 days (days 28, 29, and 30), using ultrasonic nebulization (AeroSonic; DeVilbiss, Somerset, Pennsylvania, USA).

In initial studies, no antigen-specific airway hyperresponsiveness (AHR) was induced 48 hours after 3 airway challenges with OVA. As a consequence, mice were provoked with OVA (5% in saline) (day 32) 48 hours after the last OVA challenge for 20 minutes to elicit an antigen-induced EPR and LPR (primary provocation). Airway responsiveness was determined at 5, 15, 30, and 60 minutes, and then every 30 minutes during the next 11 consecutive hours, using whole-body barometric plethysmography. In addition, numbers of eosinophils were determined in BAL on a weekly basis. Mice were reprovoked with 5% OVA when eosinophils were no longer detected in BAL and returned to the initial numbers in the lung tissue (day 74).

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**Figure 1**
Study design. BALB/c mice were sensitized by intraperitoneal injection of OVA/aluminum hydroxide on days 1 and 14. Animals were challenged for 20 minutes via the airways with 1% OVA on days 28, 29, and 30. Forty-eight hours after the last OVA challenge, mice were exposed to 5% OVA (primary provocation) for 20 minutes. AHR was determined at 5, 15, 30, and 60 minutes, and then every 30 minutes during the next 11 consecutive hours, using plethysmography. In addition, numbers of eosinophils were determined in BAL on a weekly basis. Mice were reprovoked with 5% OVA when eosinophils were no longer detected in BAL and returned to the initial numbers in the lung tissue (day 74).

**Figure 2**
Detection of EPR and LPR. After primary allergen provocation with 5% OVA, airway changes were monitored using whole-body plethysmography. The EPR was observed 5–30 minutes after the provocation (a), and was followed by the LPR 3–12 hours after initial provocation (b) (average maximum at 6 hours). Both EPRs and LPRs were observed only in sensitized and challenged animals. Neither EPRs nor LPRs were detected in sensitized only (IP; n = 12), challenged only (Neb; n = 12), or sensitized and challenged animals provoked with 5% BSA (n = 12). Response to provocation is expressed as fold change in Penh ± SEM compared with values detected after saline inhalation. *Significant differences (P < 0.05) between the groups (IPN vs. IP, Neb, and BSA). Baseline Penh_{saline} values were IPN 0.83 ± 0.03; IP 0.77 ± 0.08; Neb 0.7 ± 0.04; BSA 0.85 ± 0.09.
were provoked with saline or 5% BSA (Sigma Chemical Co.), and airway responsiveness was monitored for 12 consecutive hours to define further the specificity of the responses.

To define the pharmacological characteristics, albuterol (2.5 mg/mL solution; nebulized for 10 minutes, 10 minutes before provocation), cromolyn (20 mg/mL; nebulized for 10 minutes, 1 hour before provocation), or hydrocortisone (100 mg; nebulized for 10 minutes, 10 minutes before provocation) was used. In a separate series of experiments, anti–IL-5 (TRFK5; 60 mg/injection) was given intravenously 2 hours before primary or secondary provocation to determine the role of eosinophils in the responses; control animals received rat IgG (Figure 1).

Figure 3
Changes in RL during EPR and LPR. Airway responses to primary provocation with 5% OVA were determined using plethysmography, and are shown as changes in resistance (cm•H2O•mL−1•s ± SEM). Significant increases in RL (*P < 0.05) were observed — at 5, 15, and 30 minutes (EPR), and 6 hours (LPR) after the provocation — in the sensitized and challenged group (IPN) compared with the sensitized only (IP) and challenged only (NEB) groups (n = 12 in each group).

Figure 4
Pharmacological characterization of EPR and LPR. Sensitized and challenged mice were pretreated before primary provocation with albuterol, cromoglycate, or hydrocortisone (as described in Methods). Drugs were administered by nebulization. The EPR was blocked (*P < 0.05) by albuterol and cromoglycate, whereas the LPR was blocked (*P < 0.05) by cromoglycate and hydrocortisone (n = 8 for each group). Baseline Penh values were as follows: IPN 0.89 ± 0.1; pretreated with albuterol 0.7 ± 0.1; pretreated with cromoglycate 0.69 ± 0.12; and pretreated with hydrocortisone 0.85 ± 0.09.

Noninvasive determination of airway responsiveness to the allergen and methacholine. Airway responsiveness was assessed using a single-chamber, whole-body plethysmograph (Buxco Electronics Inc., Troy, New York, USA) (17). In this system, an unrestrained and spontaneously breathing mouse was placed into the main chamber of the plethysmograph, and pressure differences between this chamber and a reference chamber were recorded. The box pressure signal is caused by volume and resultant changes in pressure during the respiratory cycle of the animal. A low-pass filter in the wall of the main chamber allows thermal compensation. From these box pressure signals, the phases of the respiratory cycle, tidal volumes, and the enhanced pause (Penh) can be calculated. Penh is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. It correlates closely with pulmonary resistance, measured by conventional 2-chamber plethysmography in ventilated animals (17). Penh was used as a measure of airway responsiveness to allergen and methacholine (MCh).

When responsiveness to the allergen was evaluated, animals were placed in the plethysmograph, baseline values were recorded, and then mice were exposed to nebulized saline for 3 minutes to determine any nonspecific responsiveness. All responses were compared with Penhbaseline, which was taken as 1. Then animals were provoked with the allergen for 20 minutes, and airway responsiveness was measured at 5, 15, 30, and 60 minutes, and then every 30 minutes for the next 11 consecutive hours. The results of the EPR are shown in real time. For the LPR, the highest increase in Penh (after return to baseline of the EPR) was considered as the maximum of the LPR, and values recorded for 2.5 hours before and after the maximal response are shown. Responsiveness to MCh was assessed as described previously (17), before, and at 1 and 6 hours after, secondary provocation.
plethysmograph, mice were exposed for 3 minutes to nebulized PBS and subsequently to increasing concentrations of nebulized MCh (in PBS).

**Invasive determination of airway responsiveness to the allergen.** Airway responsiveness was also assessed as a change in airway function after exposure to aerosolized OVA. Anesthetized (pentobarbital sodium, intraperitoneally, 70–90 mg/kg) and tracheostomized (stainless steel cannula, 18 gauge) mice were mechanically ventilated, and lung function was assessed using methods described by Takeda et al. (13). Mice were placed in a whole-body plethysmograph and were ventilated (model 683; Harvard Apparatus Co., South Natick, Massachusetts, USA) by tracheostomy tube at 160 breaths per minute and a tidal volume of 150 mL, with a positive end-expiratory pressure of 2–4 cm H2O. Transpulmonary pressure, lung volume, and flow were determined. Lung resistance (RL) was continuously computed (LabVIEW; National Instruments, Austin, Texas, USA) by fitting flow, volume, and pressure to an equation of motion. Maximum values of RL were taken and expressed as a percentage change from baseline after saline aerosol. Measurements were performed at 5, 15, 30, and 60 minutes, and 6 hours after allergen provocation.

**Immunofluorescence.** Lungs were fixed by inflation (2 mL) and immersion in 10% formalin. Cells containing eosinophilic major basic protein (MBP) were identified by immunofluorescent staining as described previously using rabbit anti-mouse MBP. The slides were examined in a blinded fashion with a Nikon microscope equipped with a fluorescein filter system (Nikon, Garden City, New York, USA). Numbers of eosinophils in the peribronchial tissue were evaluated using the IPLab2 software (Signal Analytics Corp., Vienna, Virginia, USA) for the Macintosh, counting 6–8 different sections per animal (18).

**Determination of cell numbers and IL-5 levels in BAL.** Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with HBSS (1 mL, 37°C). Total leukocyte numbers were measured (Coulter Counter; Coulter Corp., Hialeah, Florida, USA). Differential cell counts were performed by counting at least 300 cells on cytocentrifuged preparations (Cytospin 2; Shandon Ltd., Runcorn, United Kingdom) stained with Leukostat (Fisher Diagnostics, Pittsburgh, Pennsylvania, USA) and differentiated by standard hematological procedures. BAL supernates were collected and kept frozen at −70°C. IL-5 levels were measured by ELISA. Briefly, 96-well plates (Immulon 2; Dynatech Laboratories, Chantilly, Virginia, USA) were coated with anti–IL-5 (TRFK-5) (PharMingen, San Diego, California, USA) and blocked with PBS/10% FCS overnight. Samples were added, and biotinylated anti–IL-5 (TRFK-4) was used as detection antibody; the reaction was amplified with avidin-horseradish peroxidase (Sigma Chemical Co.). IL-5 levels were determined by comparison with the known cytokine standards (PharMingen). The limit of detection was 4 pg/mL.

**Statistical analysis.** ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by Tukey-Kramer HSD test. Statistical significance was set at \( P < 0.05 \). Values for all measurements are expressed as the mean ± SEM.

**Results**

**Airway responsiveness to allergen.** Mice were sensitized and challenged with OVA as illustrated in Figure 1. On day 32, mice were reexposed (provoked) to an inhalation challenge with 5% OVA. As shown in Figure 2, allergen provocation with 5% OVA induced both an EPR and LPR. Relative increases in Penh (compared

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**Figure 5**

Peribronchial eosinophilia associated with primary OVA provocation. Eosinophil numbers in the lung were determined after staining with anti-MBP (as described in Methods). Allergen provocation led to a progressive increase in the number of tissue eosinophils that was significant (* \( P < 0.05 \)) from 15 minutes after provocation until the time of the LPR. Before allergen provocation, administration of anti–IL-5 prevented influx of eosinophils into the lung tissue, and the numbers of peribronchial eosinophils were significantly lower (\( ^{*} P < 0.01 \)) than the numbers observed in control animals that received rat IgG (\( n = 8 \) at each time point).

**Figure 6**

Levels of IL-5 in BAL after primary allergen provocation of sensitized and challenged mice on day 32. Significantly increased levels of IL-5 were observed at the time of the LPR. Before the provocation, administration of anti–IL-5 prevented the increases in IL-5, but control rat IgG had no effects on the IL-5 levels (\( n = 10 \) for each time point). \( ^{*} P < 0.01; ^{*} P < 0.05 \)
with saline, taken as 1) were observed as early as 5 minutes after the provocation (Penh 1.45 ± 0.05), reached a maximum at 15 minutes (Penh 1.7 ± 0.08), and returned to initial values by 60 minutes (Penh 1.04 ± 0.04). Early increases in Penh were only observed in those mice that were previously sensitized and challenged to OVA. Mice that were sensitized only or challenged only and then provoked, or sensitized and challenged mice provoked with saline, showed no increase in airway reactivity. Specificity was also observed, as sensitized and challenged mice provoked with BSA showed no changes in Penh. Second- or late-phase increases in Penh (1.5 ± 0.06) were observed from 3.5 to 12 hours; on average, maximum changes were observed at 6 hours after allergen provocation (mean 6 hours; median 6 hours). As in the EPR, only mice previously sensitized and challenged to OVA responded with an LPR after OVA provocation.

RL to allergen exposure was also selectively monitored using invasive techniques (Figure 3). Significant increases in RL were observed after allergen provocation at 5 minutes (0.50 ± 0.01 cmH2O•mL⁻¹s⁻¹; P < 0.05), reached their maximum at 15 minutes (0.53 ± 0.01 cmH2O•mL⁻¹s⁻¹; P < 0.05), and returned to baseline values at 60 minutes (0.41 ± 0.02 cmH2O•mL⁻¹s⁻¹). LPR-associated increases in RL were also observed 6 hours after allergen provocation (0.57 ± 0.02 cmH2O•mL⁻¹s⁻¹; P < 0.05). Mice that were sensitized but not challenged, and those not sensitized but exposed to 3 airway challenges followed by allergen provocation, did not demonstrate significant increases in RL after OVA provocation (Figure 3).

**Pharmacological modification of EPR and LPR.** Figure 4 illustrates the response to 3 agents administered before OVA provocation. Albuterol completely abolished the EPR (at 15 minutes, Penh 1.01 ± 0.03 vs. 1.90 ± 0.10 in nontreated animals; P < 0.05); however, it did not have any influence on the LPR (at 6 hours, Penh 1.50 ± 0.07 vs. 1.54 ± 0.11). Pretreatment with cromolyn abolished both the EPR (at 15 minutes, Penh 1.07 ± 0.04 vs. 1.90 ± 0.10; P < 0.05) and LPR (at 6 hours, Penh 1.08 ± 0.07 vs. 1.54 ± 0.11; P < 0.05). Administration of hydrocortisone before OVA provocation did not prevent development of the EPR (at 15 minutes, Penh 1.75 ± 0.12 vs. 1.90 ± 0.10), but significantly diminished the LPR (at 6 hours, Penh 1.17 ± 0.04 vs. 1.54 ± 0.11; P < 0.05).

**Eosinophil numbers in lung tissue after allergen provocation.** Sensitization and 3 airway challenges resulted in a significant number of eosinophils in the lung tissue and BAL, as described previously (18). Allergen provocation led to further and rapid increases in the number of tissue eosinophils, as early as 15 minutes after provocation, and reached a maximum at the time of the LPR (Figure 5). In control experiments, in which allergen provocation followed 3 airway challenges in the absence of sensitization, no further increase in numbers of eosinophils in the tissue was observed; in animals that were sensitized but not challenged, allergen provocation induced a significant increase in peribronchial eosinophilia at 3 and 6 hours; however, the number of eosinophils at 6 hours was approximately 70% less than in sensitized and challenged animals (data not shown).

**Influence of allergen provocation on IL-5 levels in BAL.** Allergen provocation led to a significant increase in IL-5 levels at 6 hours, at the time of the LPR (1,139 ± 262 pg/mL at the LPR vs. 170 ± 70 pg/mL before provocation; P < 0.01). The kinetics of IL-5 detection in BAL are shown in Figure 6.
Influence of anti–IL-5 on EPR and LPR and IL-5 levels in BAL. Administration of anti–IL-5 two hours before secondary provocation (control animals received rat IgG) prevented the LPR but not EPR. (*P < 0.01; n = 12 in each group). Baseline Penhairsine values were as follows: IPN pretreated with rat IgG 0.84 ± 0.1; IPN pretreated with anti–IL-5 0.78 ± 0.9.

Figure 8
Six weeks after the primary provocation, when the number of tissue eosinophils returned to baseline levels (day 74), mice were provoked with 5% OVA. Administration of anti–IL-5 two hours before secondary provocation (control animals received rat IgG) prevented the LPR but not EPR. (*P < 0.01; n = 12 in each group). Baseline Penhairsine values were as follows: IPN pretreated with rat IgG 0.84 ± 0.1; IPN pretreated with anti–IL-5 0.78 ± 0.9.

Discussion
Our results demonstrate that exposure of sensitized and challenged mice to allergen elicited an immediate response that reached its maximum at 15 minutes and resolved completely within 1 hour. This EPR was followed by an LPR that developed 3–12 hours later. Moreover, the early and late airway responses were allergen specific, as there were neither early nor late responses after provocation with BSA in mice previously sensitized and challenged to OVA. Increases in airway responsiveness at both the early and late phases were observed by monitoring changes in RL in anesthetized and ventilated mice, as well as by longitudinal studies using whole-body plethysmography in unrestrained, conscious animals. The EPR and LPR in this murine model also exhibited similar pharmacological characteristics as those observed previously in asthmatic patients and allergen-sensitized guinea pigs, rabbits, and sheep; the EPR was inhibited by both albuterol and cromoglycate, whereas the LPR was inhibited by cromoglycate and hydrocortisone but not by albuterol (3, 5, 10, 11, 20–22).

There have been several descriptions of both an EPR and LPR in allergen-sensitized and challenged guinea pigs (19, 20, 23, 24). In the majority of these studies, eosinophils were detected at the time of the LPR, but because of major differences in the protocols and times...
of evaluation, no causal relationships were defined. In human studies, the LPR has been associated with an increased number of eosinophils in BAL fluid and the bronchial mucosa. The kinetics of appearance and/or differences in numbers of eosinophils in the tissue are difficult to determine in humans unless repeated biopsies are done. In our model, we were able to observe the kinetics of tissue eosinophilia during development of the EPR and LPR. In all of our studies, eosinophils were present at the time of the LPR. In the initial studies, in which allergen provocation closely followed allergen sensitization and challenge, eosinophil numbers in the lung and BAL were already increased before allergen provocation, as a result of previous challenge of sensitized mice. Monitoring eosinophil numbers in the tissue in peribronchial areas revealed significant increases after allergen provocation, which reached their maximum at the time of the LPR. Moreover, the LPR was associated with a significant increase in IL-5 detected in BAL. It has been difficult for us to measure tissue levels of IL-5, preventing us from correlating tissue levels of IL-5 with tissue eosinophil numbers. Interestingly, the levels of IL-5 in BAL were initially detected after eosinophil numbers had already begun to increase in the tissue. This apparent delay or dissociation between tissue and BAL eosinophils is typical of this model (see later here). In animals that were challenged only, or sensitized but not challenged, IL-5 levels in BAL after provocation were below the detection level at the time of the LPR (6 hours after provocation) (data not shown). Administration of anti–IL-5 2 hours before provocation prevented both the enhanced recruitment of eosinophils to the peribronchial regions and the increases in IL-5 levels at the time of the LPR. This pretreatment was associated with the failure of LPR development without affecting the EPR.

To further correlate tissue eosinophils with the development of the LPR, while eliminating the persistent influence of sensitization and challenge on eosinophil numbers, sensitized and challenged mice were provoked with allergen only after eosinophil numbers in the lung tissue were restored to baseline numbers, approximately 6 weeks after provocation. Provocation with allergen at this time point (day 74) led to significant and progressive increases in peribronchial eosinophilia, beginning at 3 hours and increasing through the peak of the LPR. At these time points, eosinophils were not detected in the BAL. Preventing these increases in eosinophil numbers by administering anti–IL-5 before provocation completely inhibited development of the LPR, whereas the EPR was unaffected. These studies associate eosinophils with the development of the LPR, and demonstrate the independence of the EPR on eosinophilic inflammation. Furthermore, these findings illustrate the correlation of tissue eosinophilia, but not BAL eosinophilia, with AHR at the time of the LPR.

To our knowledge, we have for the first time characterized and compared the requirements for development of the EPR and LPR in a murine model of allergen-induced AHR. These studies were carried out and the results confirmed after both primary and secondary provocation of sensitized mice. We demonstrated that the EPR and LPR have distinguishable pharmacological features that are similar to those observed in human asthma. Allergen provocation resulted in a significant influx of eosinophils into the lung tissue that, after provocation, was very rapid (within the first hour); provocation also resulted in an increase in IL-5 in BAL that coincides with the LPR but not the EPR. Inhibition of this influx of eosinophils, as demonstrated with anti–IL-5, was associated with inhibition of the LPR while leaving the EPR intact. These findings indicate an essential role for IL-5 and tissue eosinophils in the development of the LPR in this murine model of asthma. This ability to detect and distinguish the EPR and LPR in a murine model will enable us to characterize further the key features of each reaction, as well as the response to different interventions.

**Figure 9**
Effect of anti–IL-5 on tissue eosinophilia. Six weeks after the primary provocation, only small numbers of eosinophils were detected in the lung tissue. Animals were exposed to 5% OVA (secondary provocation) that led to significant (*P < 0.05) increases in the number of eosinophils in the lung tissue at the time of LPR. Administration of anti–IL-5, but not rat IgG, prevented the influx of eosinophils into the lung (*P < 0.01) (n = 8 at each time point).

**Figure 10**
Airway responsiveness to inhaled MCh. On day 74, six weeks after primary provocation, airway responsiveness to inhaled MCh was assessed before, and at 1 and 6 hours after, secondary provocation with 5% OVA in sensitized and challenged mice. Baseline Penh values were 0.71 ± 0.04 for naive animals and 0.8 ± 0.03 for sensitized and challenged mice (*P < 0.05).
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