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Induction of a late asthmatic response associated with airway inflammation in mice

Takeshi Nabe [a,b], Carlene L. Zindl [a], Yong Woo Jung [c], Robin Stephens [a,1], Akari Sakamoto [b], Shigekatsu Kohno [b], T. Prescott Atkinson [d], David D. Chaplin [a,*]

[a] Department of Microbiology, University of Alabama at Birmingham, 859 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294-2170, USA
[b] Department of Pharmacology, Kyoto Pharmaceutical University, 5 Nakauchi, Misasagi, Yamashina, Kyoto 607-8414, Japan
[c] Department of Pathology, University of Alabama at Birmingham, 859 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294-2170, USA
[d] Department of Pediatrics, University of Alabama at Birmingham, 614 Ambulatory Care Center, 1600 7th Avenue South, Birmingham, AL 35233-1711, USA

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Abstract

To investigate mechanisms underlying the late asthmatic response, we developed a murine model using repetitive intratracheal antigen challenge. BALB/c mice sensitized by i.p. injection with ovalbumin+alum were challenged with ovalbumin intratracheally 4 times. The 1st challenge induced early airway obstruction peaking at 30 min but without a late response; however, the 4th challenge caused not only early but also late airway obstruction at 2–8 h. Eosinophils, and $CD4^+$ and $CD8^+$ T lymphocytes were increased in the airway before the 4th but not before the 1st–3rd challenges. The numbers of $IgE^+/CD117^+$ (mast) cells were also increased in the lung before the 4th challenge. Levels of Th2 cytokines were also increased in the airway. Daily administration of dexamethasone during the challenge period suppressed all these inflammatory events. Thus, this experimental late asthmatic response is associated with Th2 cytokine production from inflammatory cells recruited as a consequence of the 1st–3rd challenges.

Keywords: Asthma; Late phase response; Mast cell; Inflammation; Corticosteroid; Cytokine

1. Introduction

Allergic asthma in humans is characterized by airway inflammation with a prominent eosinophil component, airway hyperresponsiveness to non-specific stimuli, and allergen-induced early and late asthmatic responses (Herxheimer, 1952; Cockcroft et al., 1977; Durham, 1991). The early asthmatic response is characterized by acute airway obstruction beginning within a few min after allergen exposure and resolving over the next 60–120 min. The late asthmatic response, in contrast, begins 60–90 min after antigen exposure and persists for more than 4 to 6 h. Animal models showing early and late asthmatic responses have been developed using guinea pigs (Iijima et al., 1987; Hutson et al., 1988; Nabe et al., 1997a, 1998a), rats (Waserman et al., 1992) and sheep (Abraham et al., 1983). Mice provide excellent models to analyze mechanisms of inflammatory diseases because of the many immunological tools available in this system. In asthma research using mice, however, most researchers have focused on airway hyperresponsiveness and lung eosinophilia as the major physiological parameters. In spite of its importance in human asthma, the late asthmatic response has not been well studied in mice.

Cieslewicz et al. (1999) first reported a murine model in which BALB/c mice sensitized i.p. with ovalbumin adsorbed to alum and challenged repeatedly with aerosolized ovalbumin showed not only early but also late asthmatic responses. The

* Corresponding author. 276 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294-2170, USA. Tel.: +1 205 934 9339; fax: +1 205 934 9256.
E-mail address: dchaplin@uab.edu (D.D. Chaplin).
[1] Current address: Division of Parasitology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

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experimental early asthmatic response was inhibited by treatment with a β_2-adrenoceptor stimulatory bronchodilator but not with a corticosteroid (Cieslewicz et al., 1999). In contrast, the late asthmatic response was inhibited by treatment with a corticosteroid, a neutralizing anti-interleukin (IL)-5 monoclonal antibody, or an antisense IL-5 oligonucleotide but not by treatment with a β_2-stimulant (Cieslewicz et al., 1999; Karras et al., 2000). Thus, the murine late asthmatic response could represent airway obstruction that is a consequence of eosinophil predominant airway inflammation rather than simply a consequence of mediator-induced smooth muscle constriction. Additionally, in another recent study, involvement of IL-13 in the late asthmatic response was established because treatment with a soluble IL-13 receptor α2-IgG fusion protein attenuated the response (Taube et al., 2002); however, other mechanisms that might contribute to the induction of the late asthmatic response were not defined.

Because we intended to focus this study on the mechanisms of the asthmatic response, we analyzed antigen challenge-induced changes in airway resistance in conscious animals. Although we recognize that there may be important interactions between the upper airway and the lower airway in developing the full asthmatic phenotype, our intent in this study was to measure responses that were induced specifically in the lung, with as little contribution as possible from responses in the nasal cavity. Most researchers have used aerosolized antigen or intranasal antigen challenge to elicit airway hyperresponsiveness, and early and late asthmatic responses; however, using these types of challenges, the antigen passes through the nasal cavity before being delivered to the lung, and the dose delivered to the lung is unpredictable. Intranasal application of antigen in systemically sensitized mice can induce local allergic responses including eosinophil recruitment to the nasal tissue (Malm-Erjefalt et al., 2001; Matsui et al., 2000; Saito et al., 2002; Hussain et al., 2001). Although to our knowledge there have been no reports of a murine allergic rhinitis model exhibiting nasal congestion and reduced nasal air flow, patency of the nasal airway of sensitized guinea pigs challenged with intranasal antigen (Narita et al., 1997; Nabe et al., 1998b) is known to be reduced. Consequently, to assure that the changes we measured in airway physiology in these studies represent changes in lung airflow, we delivered the challenge antigen directly into the trachea, bypassing the nasal cavity and pharynx.

The primary goal of this study was to investigate the pathophysiology of the late asthmatic response using a reproducible experimental model based on multiple intratracheal (i.t.) antigen challenges in sensitized mice. We structured the experiments to relate the induction of late asthmatic response to histological changes, increases of airway leukocytes including eosinophils, $CD4^+$ T cells, $CD8^+$ T cells and mast cells, cytokine production profiles in the lung, and antigen-specific immunoglobulin accumulation in the serum. Lastly, we investigated the effects of the corticosteroid, dexamethasone, on changes in these parameters. Our findings define a reliable protocol for establishment of the late asthmatic response in mice, indicate an association between the late asthmatic response and airway inflammation, and demonstrate that treatment with dexamethasone can block the late asthmatic response in this animal model.

2. Materials and methods

2.1. Sensitization and challenge

Six-week old BALB/c mice purchased from Harlan Sprague Dawley Inc., (Indianapolis, IN) and Japan SLC (Hamamatsu, Japan) were sensitized by i.p. injection with ovalbumin (Grade V, Sigma Chemical Co., St. Louis, MO) adsorbed to alum, which was made by us as previously reported (Nabe et al., 1997b). Ovalbumin was used at a dose of 50 μg adsorbed to 2 mg alum/0.5 ml of phosphate buffered saline (PBS)/animal on days 0 and 14. Non-sensitized control mice were injected with 2 mg alum in PBS alone. Both the sensitized and non-sensitized mice were challenged on days 28, 29, 30 and 33 under inhalation anesthesia with isoflurane (Halocarbon Lab., River Edge, NJ) with 2% ovalbumin at a volume of 25 μl by i.t. administration as reported previously (Ho and Furst, 1973).

When indicated, dexamethasone (3 mg/10 ml of PBS/kg; dexamethasone 21-phosphate disodium salt, Sigma Chemical Co., St. Louis, MO) was administered i.p. daily on days 28–33 (designated 'successive treatment'). On days 28, 29, 30 and 33, dexamethasone was administered 2 h before the i.t. antigen challenge. In another group of mice, dexamethasone was injected only once 2 h before the 4th i.t. antigen challenge (designated 'single treatment').

This animal study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham, and the Experimental Animal Research Committee at Kyoto Pharmaceutical University. It was also fully in compliance with the European Community guidelines for the used of experimental animals. Mice were kept in microisolator cages and provided sterile food and water ad libitum.

2.2. Measurement of pulmonary function

As an indicator of airway resistance, Penh (enhanced pause) was estimated at the indicated times after the 1st–4th ovalbumin challenges using a whole-body plethysmography system (Buxco Electronics, Troy, NY) as previously described (Hamelmann et al., 1997). In selected experiments, specific airway resistance (sRaw, $cmH_2Oxml/(ml/sec)$) was measured before and after the 4th ovalbumin challenge using a double-flow plethysmography system (Pulmos-I. II. III, M.I.P.S., Osaka, Japan) according to the method of Pennock et al. (1979) and Flandre et al. (2003).

Both the whole body and the double-flow plethysmography systems are non-invasive technologies allowing us to assess pulmonary function longitudinally over a prolonged time course. Having the ability to make repeated measurements was essential for analysis of the late asthmatic response. Analysis of pulmonary function using invasive methods that require catheterization of the trachea in anesthetized mice is impractical for measurement of the late asthmatic response since this response evolves over a 6–8 h time course. Reliability and

reproducibility of measurements made using whole body and double-flow plethysmography were increased by assuring that all measurements were made in an environment controlled for temperature (74–76 °F) and humidity (50–60%).

2.3. Analysis of cells recovered by bronchoalveolar lavage (BAL)

Before and 4 h or 2 d after the indicated airway antigen challenges, mice were sacrificed by lethal injection using 50 μl of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). The pulmonary circulation was perfused using 5 ml PBS, and then the lung was lavaged via a tracheal catheter using two aliquots of 0.8 ml PBS containing 2% fetal bovine serum. Total leukocyte numbers were counted with a hemacytometer. To determine the differential cell counts, BAL cells were treated with ACK lysis buffer to remove contaminating erythrocytes and centrifuged onto a glass slide by Cytospin 3 (Shandon, PA) at 50 ×*g* for 30 s at 4 °C followed by staining with Diff-Quik solution (Dade Behring Inc., Deerfield, IL).

Numbers of $CD4^+$ T cell and $CD8^+$ T cells in BAL fluid were measured by flow cytometry. In brief, BAL cells were first incubated with anti-mouse FcγRII/III antibody (clone 2.4G2, BD PharMingen, San Diego, CA) for 20 min at 4 °C to prevent binding of subsequent antibodies to FcγRII/III. The cells were then incubated with phycoerythrin (PE)-labelled monoclonal anti-mouse CD8α antibody (Ly-2) (clone 53-6.7) and with Cy-Chrome-labelled monoclonal anti-mouse CD4 antibody (L3T4) (clone H129.19) (both from BD PharMingen) at 1–4 μg/ml for 20 min at 4 °C. After washing three times with PBS supplemented with 2% fetal bovine serum, the stained cells were fixed with 4% paraformaldehyde for 12–18 h, and then analysed using a FACSCalibur (Becton Dickinson, San Jose, CA) and Cell Quest software (version 3.3, Becton Dickinson).

2.4. Detection of IgE^+, $CD117^+$ cells and IgE^+, $CD117^-$ cells in the lung

We determined the numbers of mast cells recovered from lung tissue using flow cytometry to assess surface IgE and CD117 as described by Yamaguchi et al. (1997). The left upper lobes of the lungs were isolated, finely minced in PBS supplemented with 2% fetal bovine serum, and then forced through 40-μm pore size nylon screens. The resulting single cell suspensions were washed by centrifugation and the numbers of live cells were determined by trypan blue exclusion following treatment with ACK lysing buffer to remove erythrocytes. In selected experiments, numbers of dead cells were estimated by flow cytometry after staining with 7-aminoactinomycin D, with similar results. Isolated cells were first incubated with both monoclonal anti-mouse FcγRII/III antibody (clone 2.4G2) and anti-mouse CD23 antibody (clone B3B4, BD PharMingen) for 15 min at 4 °C to block binding of subsequent antibodies to FcγRII/III and FcεRII, respectively. After washing once with PBS supplemented with 2% fetal bovine serum, the cells were incubated at 4 °C with a monoclonal mouse IgE anti-DNP antibody (clone SPE-7, Sigma Chem., St. Louis, MO) at 10 μg/ml for 50 min to saturate fully FcεRI on the lung cells, and then, simultaneously for the last 25 min, with PE monoclonal anti-mouse CD117 (c-kit) antibody (clone 2B8, BD PharMingen) at 4 μg/ml. After washing once with PBS supplemented with 2% fetal bovine serum, the cells were stained with FITC-labelled monoclonal anti-mouse IgE antibody (clone R35-72, BD Pharmingen) at 10 μg/ml for 25 min at 4 °C. After washing 3 times, the stained cells were incubated with 7-aminoactinomycin D (Molecular Probes, Eugene, OR) at 1 μg/ml for more than 30 min at 4 °C. Dead cells that were positive for 7-aminoactinomycin D were excluded from analysis. At least 10,000 live cells in each sample were analyzed using the FACSCalibur and Cell Quest.

2.5. Histological studies

The left lower lobe of the lung was isolated from 24 sensitized mice 2 h before the 1st, 2nd, 3rd and 4th challenges (6 animals each). The isolated lung was fixed with 10% formalin, and then the tissues were embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin for light microscopic examination.

2.6. Measurement of titers of ovalbumin-specific IgE and IgG1 antibodies

Serum levels of ovalbumin-specific IgE and IgG1 antibodies were determined by ELISA using samples collected just before each of the i.t. antigen challenges using methods reported previously (Kung et al., 2001). Values for serum ovalbumin-specific IgE and IgG1 levels were expressed in arbitrary units relative to a reference sample of pooled sera from the sensitized-challenged mice.

2.7. Measurement of cytokines and eotaxin

Levels of IL-4, IL-5, IL-10, IL-13, interferon (IFN)-γ and eotaxin in BAL fluids were measured using quantitative colorimetric sandwich ELISA kits (R and D Systems, Minneapolis, MN).

2.8. Statistical analyses

Statistical analyses were performed by one-way analysis of variance. If significant differences were detected, individual group differences were determined by a Bonferroni–Dunn test. Comparison between values of pre- and post-challenge was performed by paired *t*-test. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Time-course of changes in Penh after i.t. ovalbumin challenge

In order to define the relationship between airway antigen challenge, and early and late asthmatic responses, we

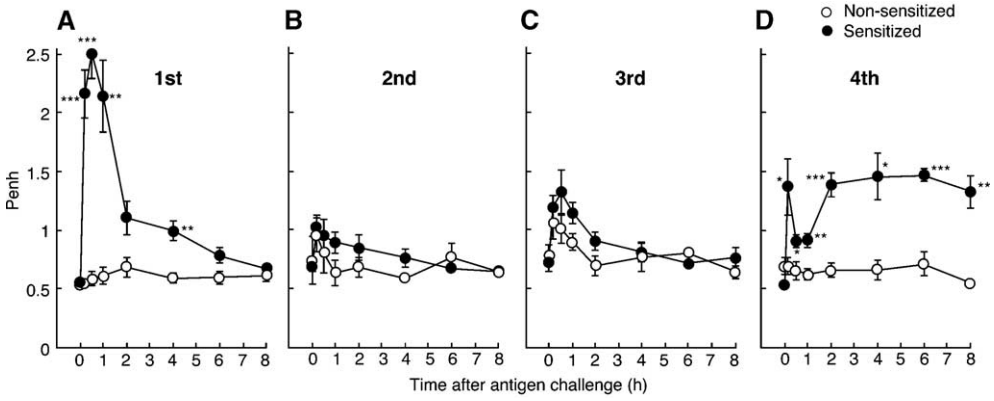

Fig. 1. Time-course of changes in Penh after i.t. antigen challenge. Changes in Penh after the (A) 1st, (B) 2nd, (C) 3rd and (D) 4th i.t. ovalbumin challenge in non-sensitized and sensitized mice are shown. Sensitized mice received an i.p. injection with ovalbumin plus alum on days 0 and 14, and non-sensitized mice received alum alone. Antigen challenges were by i.t. administration of ovalbumin solution on days 28 (1st), 29 (2nd), 30 (3rd) and 33 (4th). Each data point represents mean Penh±S.E.M. of 4 (non-sensitized) and 8 (sensitized) animals. *, ** and ***: $P<0.05$, 0.01 and 0.001, respectively, vs. non-sensitized mice.

investigated the time-course of changes in Penh after the 1st, 2nd, 3rd and 4th i.t. ovalbumin challenges. The 1st challenge induced a prominent increase in Penh over the period from 10 min to 1 h after the challenge. This represented the early asthmatic response. The Penh value gradually returned to the baseline value by the 6th–8th h after the challenge (Fig. 1A). Interestingly, the 2nd and 3rd challenges induced either no detectable or a profoundly decreased early asthmatic response (Fig. 1B and C). In contrast, the 4th challenge, performed 3 d after the 3rd challenge, induced an early asthmatic response peaking at the 10th min. The Penh then decreased over the period from 30 min to 1 h after the challenge before increasing again beginning at the 2nd h and extending at a plateau through the 8th h at levels of 250–300% of the baseline (Fig. 1D). This late increase in Penh, defining a late asthmatic response, was not observed in non-sensitized mice. Penh values before, 10 and 30 min, and 1, 2, 4, 6 and 8 h after the 4th challenge were 0.535±0.017, 1.371±0.238*, 0.907±0.057***, 0.914±0.055***, 1.389±0.102***, 1.458±0.200**, 1.468±0.055***, and 1.330±0.140** ($N=8$, *, ** and ***: $P<0.05$, $P<0.01$ and $P<0.001$ vs. the value before the challenge), respectively.

To verify that the observed increases in Penh represented increases in airway resistance during the early and late asthmatic responses, we measured changes in sRaw over time after the 4th antigen challenge. As shown in Fig. 2, almost identical to the time-course pattern of the changes in Penh, sRaw also showed a biphasic increase, peaking at 10 min and again between 4 and 8 h. Thus, changes in Penh were similar to changes in airway resistance during the early and late asthmatic responses in this model. SRaw values before, and 10 min, 1, 2, 4, 6 and 8 h after the 4th challenge were 2.717±0.047, 3.994±0.372**, 2.658±0.166, 2.946±0.231, 3.444±0.292*, 3.376±0.278*, and 3.470±0.301* ($N=10$, * and **: $P<0.05$ and $P<0.01$ vs. the value before the challenge), respectively.

3.2. Time-course of production of ovalbumin-specific IgE and IgG1 antibodies

To examine the relationship between the production of serum ovalbumin-specific IgE and IgG1 antibodies and the induction of the early and late asthmatic responses, we assessed the time-course of changes in these parameters relative to the i.t. ovalbumin challenges. Serum titers of ovalbumin-specific IgE antibody were increased after i.p. immunizations with ovalbumin plus alum, but then were decreased after the first airway challenge, measured prior to the 2nd and 3rd challenges. Serum IgE anti-ovalbumin was again increased by the time of the 4th challenge (Fig. 3A). There were increased levels of serum ovalbumin-specific IgG1 antibody that were not significantly changed at the 1st, 2nd, or 3rd challenges. Before the 4th challenge, ovalbumin-specific IgG1 antibody titers were further increased (Fig. 3B). These data suggest that because elevated IgE levels were present prior to the first challenge, this isotype alone is not sufficient to drive the late asthmatic response. There was, in contrast, a correlation between highly increased IgG1 antibodies and the development of the late asthmatic response, consistent with the possibility that a high level of IgG antibodies may contribute to the development of the late asthmatic response, perhaps via an Arthus-type reaction.

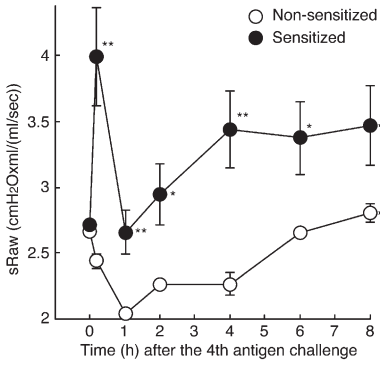

Fig. 2. Time-course of changes in sRaw after the 4th antigen challenge. Mice were sensitized to ovalbumin as in Fig. 1 and were challenged with the same antigen by i.t. instillation. Changes in sRaw after the 4th i.t. ovalbumin challenge in non-sensitized and sensitized mice are shown. Each data point represents mean sRaw±S.E.M. of 8 (non-sensitized) and 10 (sensitized) animals. * and **: $P<0.05$ and 0.01, respectively, vs. non-sensitized mice.

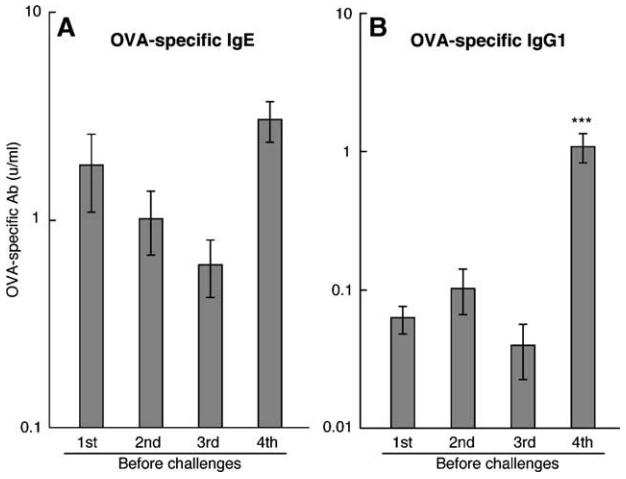

Fig. 3. Time-course of changes in the levels of ovalbumin-specific IgE and IgG1 antibodies relative to intratracheal ovalbumin challenges. Sera were collected from ovalbumin-sensitized mice 2 h prior to each of the 1st, 2nd, 3rd and 4th i.t. antigen challenges and ovalbumin-specific IgE (panel A) and IgG1 (panel B) antibody levels were determined by ELISA. One u/ml ovalbumin-specific antibody was defined based on values for pooled immune sera. Each data point represents the mean antibody concentration ± S.E.M. of 7–9 animals. ***: $P<0.001$, vs. the value before the 1st challenge.

3.3. Time-course of recruitment of leukocytes to the airways

To investigate the nature of the inflammatory changes that had been induced at the time when the late asthmatic response could be elicited (the 4th challenge), we determined the time-course of leukocyte recruitment into the lungs and airways following antigen challenge of ovalbumin-sensitized mice. We combined observation of photomicrographs of histological sections (Fig. 4) to demonstrate the organization of the inflammatory cell infiltrates with quantitative analysis of airway inflammation using flow cytometric analysis of cells harvested by BAL (Fig. 5). As defined by histological analysis using hematoxylin and eosin staining, almost no leukocyte recruitment was induced before the 1st or the 2nd airway challenges (Fig. 4A and B). Two h before the 3rd challenge (2 and 1 d after the 1st and 2nd challenges, respectively), inflammatory cells were minimally but consistently increased (Fig. 4C). In contrast, there was marked infiltration of leukocytes around the blood vessels and airways prior to the 4th challenge (3 d after the 3rd challenge) (Fig. 4D). There was no detectable further increase in inflammatory cells either 4 h or 2 d after the 4th challenge (data not shown).

In order to characterize the nature and quantity of inflammatory cells that were recruited as a result of airway challenge, we also performed BAL. Fig. 5 shows the time-course of the changes in numbers of leukocytes recovered in BAL fluid before and after each of the airway challenges. All leukocytes analyzed except for neutrophils showed a similar pattern of infiltration into the lung. Mononuclear cells (macrophages and lymphocytes), eosinophils, $CD4^+$ T cells, and $CD8^+$ T cells all were recovered in the BAL in minimal numbers prior to and following the first 3 antigen challenges. In contrast, all cell types except for neutrophils were dramatically increased prior to the 4th challenge. In addition, the 4th challenge did not induce immediate (by 4 h after challenge) further recruitment of leukocytes to the airways, although there was further increased recruitment of $CD4^+$ T cells, $CD8^+$ T cells and eosinophils 2 d after the 4th i.t. ovalbumin challenge (Fig. 5A, B, C and D).

Neutrophils showed a different pattern of recruitment to the airways. Even in non-sensitized mice, administration of ovalbumin i.t. induced neutrophil recruitment following each

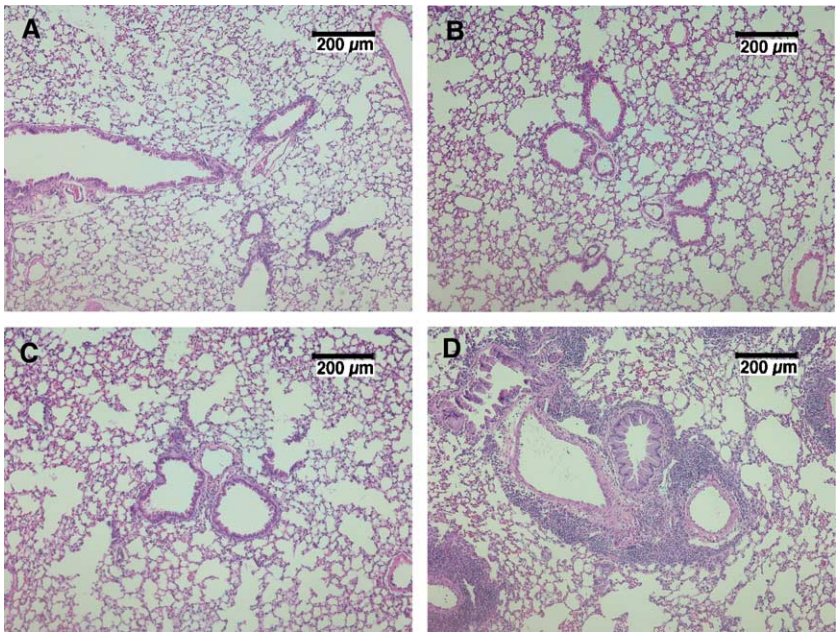

Fig. 4. Development of lung inflammation following airway antigen challenge. Mice were sensitized to ovalbumin as in Fig. 1 and were challenged with the same antigen by i.t. instillation. Two h before the 1st (panel A), 2nd (panel B), 3rd (panel C) and 4th (panel D) challenge, lung tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin. Similar data were obtained in 5 additional experiments.

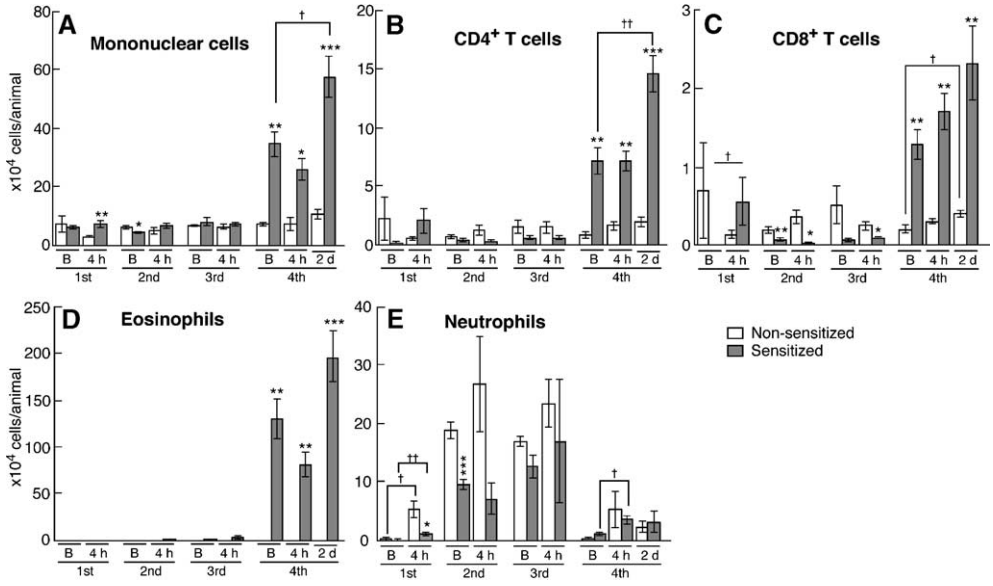

Fig. 5. Time-course of leukocyte inflammation as assessed by BAL. Data shown represent changes in numbers of total mononuclear cells (panel A), $CD4^+$ T cells (panel B), $CD8^+$ T cells (panel C), eosinophils (panel D) and neutrophils (panel E) in BAL fluid harvested 2 h before (B) and 4 h or 2 d after the 1st, 2nd, 3rd and 4th challenges in non-sensitized and sensitized mice. Each bar represents the mean number of cells±S.E.M. of 4–6 animals. *, ** and ***: $P<0.05$, 0.01 and 0.001 vs. non-sensitized; and † and ††: $P<0.05$ and 0.01, respectively.

of the first three airway challenges. Interestingly, the magnitude of the ovalbumin-induced airway neutrophilia in sensitized mice was consistently smaller than that in non-sensitized mice at the time points measured. By 2 h prior to the 4th challenge, most of the previously recruited neutrophils had disappeared, and the 4th challenge induced only a small increase of neutrophils in both sensitized and non-sensitized mice (Fig. 5E). The relatively low numbers of neutrophils in the inflammatory infiltrates argues against an Arthus-type mechanism as an explanation for the late asthmatic response after the 4th airway challenge of sensitized mice (Williams et al., 1986).

3.4. Time-course of changes in the production of Th1 and Th2 cytokines and eotaxin

To investigate the relationship between key cytokines produced as a consequence of the i.t. antigen challenge and the induction of the late asthmatic response, we measured levels of the Th1 cytokine IFN-γ, the Th2 cytokines IL-4, IL-5 and IL-

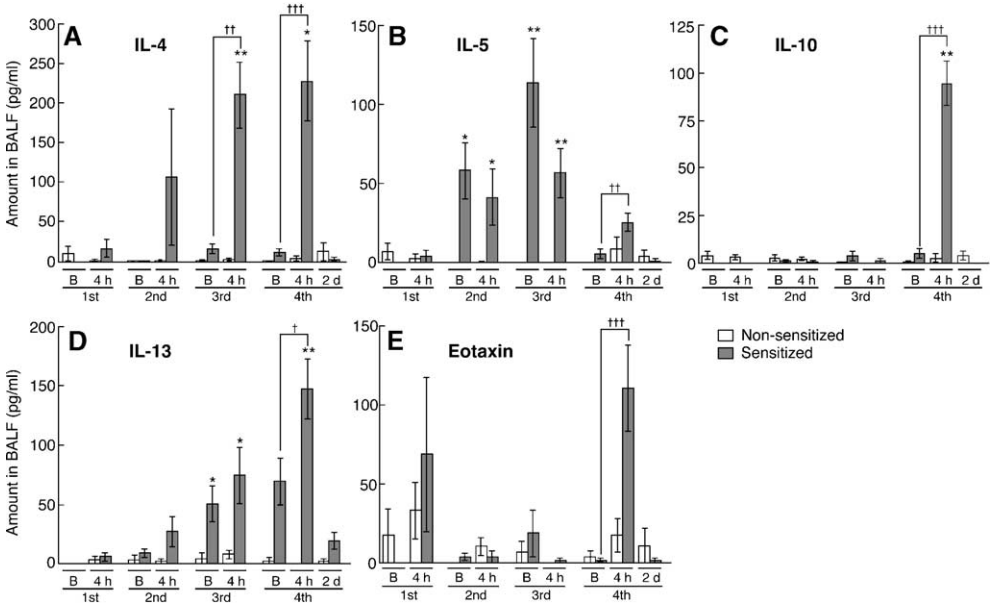

Fig. 6. Antigen-induced production of airway cytokines and eotaxin. Mice sensitized with ovalbumin as in Fig. 1 were challenged i.t. BAL fluid was recovered either 2 h before (B) or 4 h (4 h) after each challenge, and 2 d after the 4th challenge, and IL-4 (panel A), IL-5 (panel B), IL-10 (panel C), IL-13 (panel D) and eotaxin (panel E) were measured by ELISA. Bars represent mean concentration of cytokine±S.E.M. of 4–6 non-sensitized and sensitized mice. * and **: $P<0.05$ and 0.01 vs. non-sensitized; and †, †† and †††: $P<0.05$, 0.01 and 0.001, respectively.

13, the immunomodulatory cytokine IL-10, and the chemokine eotaxin in BAL fluid before and after each of the i.t. antigen challenges. Except for low levels of airway eotaxin recovered following the first i.t. challenge (Fig. 6E), these cytokines were not detected in non-sensitized mice that received ovalbumin challenge. Although IL-4 levels were at baseline immediately prior to each challenge, increased levels of IL-4 were detected in BAL fluid collected 4 h after each challenge of sensitized mice. By 2 d after the 4th challenge, IL-4 levels had returned to baseline (Fig. 6A). IL-5 levels were markedly increased before and 4 h after the 2nd and 3rd challenges of sensitized mice. Unexpectedly, the magnitude of the increase in IL-5 detected before and 4 h after the 4th challenge was smaller than that observed for the 2nd and 3rd challenges (Fig. 6B) suggesting that a counter-regulatory mechanism had been induced by this latter time point. A detectable increase in IL-10 was induced only after the 4th challenge, and this increase was short-lived (Fig. 6C). Levels of airway IL-13 gradually increased with each challenge and remained persistently elevated prior to each subsequent challenge (Fig. 6D). In contrast, IFN-γ levels increased only minimally before or after any of the challenges (<5 pg/ml; data not shown). Eotaxin was increased 4 h after the 1st and 4th challenges (Fig. 6E).

3.5. Inhibition of the asthmatic response and lung inflammation by dexamethasone

To test whether the late asthmatic response in this model is inhibited by systemic treatment with a corticosteroid, we analyzed the effects of treatment with a single dose or with multiple successive doses of dexamethasone. The induction of the late asthmatic response was almost completely suppressed by successive dosing but not by a single dose of dexamethasone administered 2 h prior to the 4th antigen challenge. The early asthmatic response was also strongly inhibited by the successive dosing treatment (Fig. 7). Serum ovalbumin-specific IgE levels in groups of mice treated using the successive dosing regimen with either the vehicle control (PBS) or dexamethasone were 3.12±0.99 (N=8) and 2.65 ±0.89 (N=7) (u/ml), respectively. Ovalbumin-specific IgG1 levels in these 2 groups were 0.50±0.07 (N=8) and 0.48 ±0.13 (N=7) (u/ml), respectively. Numbers of eosinophils, total mononuclear cells (MNC), $CD4^+$ T cells and $CD8^+$ T cells that had been recruited to the lung 4 h after the 4th challenge were significantly suppressed by the successive dosing but not by the single treatment dexamethasone regimen (Table 1). The low level of neutrophil recruitment seen following the 4th challenge was unaffected by either dexamethasone dosing regimen. The increased levels of IL-4, IL-10, IL-13 and eotaxin detected 4 h after the 4th antigen challenge were also strongly suppressed by the successive dosing regimen. Single dose treatment with dexamethasone did not significantly affect the production of either cytokines or eotaxin (Table 2).

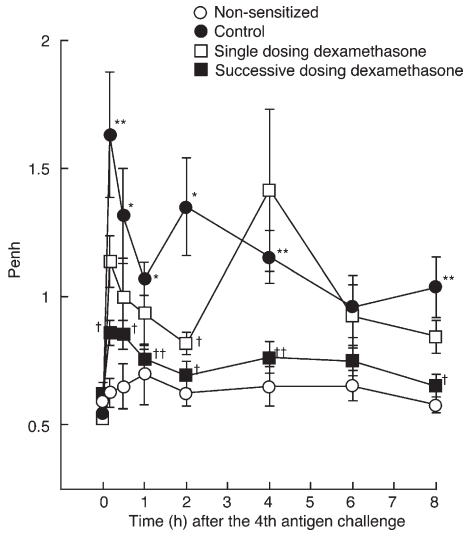

Fig. 7. The successive dosing dexamethasone regimen blunts both the early and late asthmatic responses. Naïve mice were injected i.p. with ovalbumin plus alum (Control Sensitized) or with alum alone (non-sensitized), then challenged four times with ovalbumin i.t. Sensitized and challenged mice were treated using the single dosing dexamethasone (3 mg/kg/dose) regimen (1 dose 2 h before the 4th antigen challenge) or the successive dosing regimen (1 dose 2 h before each of the i.t. antigen challenges and once a day between the 3rd and 4th challenges). Each point represents mean Penh±S.E.M. of 5–7 animals at the indicated times following the 4th antigen challenge. *, ** and ***: P<0.05, 0.01 and 0.001 vs. non-sensitized; and †, †† and †††: P<0.05, 0.01 and 0.001, respectively, vs. control.

Table 1
The successive dose dexamethasone regimen inhibits leukocyte recruitment to the airways

	×10^4 cells/animal				
	MNC	$CD4^+$	$CD8^+$	Eos	Neut
Control	25.2±5.6	6.1±0.9	1.3±0.3	76.4±18.0	2.9±0.9
Dexamethasone (single)	22.2±3.9	3.7±0.8	0.7±0.2	56.4±10.2	2.8±0.8
Dexamethasone (successive)	11.3±1.7[a]	1.3±0.3[b]	0.2±0.1[b]	29.1±8.4[a]	3.8±1.1

Mice were sensitized as in Fig. 7, and then challenged i.t. four times with ovalbumin. Cell recruitment was analyzed by BAL 4 h after the final challenge. Dexamethasone (3 mg/kg/dose) was administered once 2 h prior to the 4th challenge (single) or daily with the successive dosing regimen. Each value represents the mean±S.E.M. of 8 or 9 animals. [a] and [b]: P<0.05 and <0.001, respectively, vs. control. MNC, mononuclear cells; $CD4^+$, $CD4^+$ T cells; $CD8^+$, $CD8^+$ T cells; Eos, eosinophils; Neut, neutrophils.

Table 2
Treatment with dexamethasone blunts the airway cytokine response

	pg/ml				
	IL-4	IL-5	IL-10	IL-13	Eotaxin
Control	195±42	35±8	93±12	169±37	118±44
Dexamethasone (single)	174±53	24±11	55±17	92±31	34±19
Dexamethasone (successive)	22±4[b]	5±2[b]	20±5[b]	10±2[b]	14±6[a]

Sensitized and challenged mice were treated with dexamethasone using either the single dose or the successive dosing regimen and were compared to control mice that received no dexamethasone. BAL samples were harvested 4 h after the last challenge, and levels of IL-4, IL-5, IL-10, IL-13 and eotaxin were measured by ELISA. Each value represents the mean cytokine concentration±S.E.M. of 8 or 9 animals. [a] and [b]: P<0.05 and <0.01, respectively, vs. control.

3.6. Modulation of mast cell accumulation in the lung by dexamethasone

Based on our observation here that treatment with dexamethasone blocked induction of the early asthmatic response, and the previously demonstrated ability of β_2-adrenoreceptor agonists to block the early asthmatic response (Cieslewicz et al., 1999), we speculated that the numbers of lung mast cells might have been increased by the multiple airway antigen challenges and that dexamethasone might inhibit this mast cell hyperplasia. Consequently, we investigated the numbers of lung mast cells (IgE^+, $CD117^+$ cells) using flow cytometry. As shown in Fig. 8B and C, IgE^+, $CD117^+$ cells could be detected in suspensions of cells from lungs of sensitized mice harvested before the 4th challenge ($2–3 \times 10^3$ cells per left upper lobe). Small numbers of IgE^+, $CD117^+$ cells ($<0.1 \times 10^3$ per left upper lobe) were detected in the non-sensitized mice even after multiple i.t. ovalbumin challenges (Fig. 8A and C). In the sensitized mice, the numbers of mast cells were increased above non-sensitized mice by the time of the 3rd challenge, with a further increase observed before and 4 h after the 4th challenge (Fig. 8C). Thus, the ability to manifest a late asthmatic response was correlated with increased numbers of IgE^+, $CD117^+$ mast cells in the lungs. Interestingly, in addition to the IgE^+, $CD117^+$ mast cells, there were large numbers of IgE^+, $CD117^-$ cells in the lung tissue of sensitized and challenged mice (Fig. 8B and D). Prior studies have reported that IgE^+, $CD117^-$, I-A/I-E^- cells in murine lung are basophils (Luccioli et al., 2002), and that some subsets of antigen presenting cells, such as peripheral blood dendritic cells, have FcεRI on the cell surfaces and are expected to be IgE^+ (Novak et al., 2003). Thus, the IgE^+, $CD117^-$ cells we have observed in suspensions of lung cells are likely to consist of a mixture of basophils and antigen presenting cells. IgE^+, $CD117^-$ cells were substantially increased by the time of the 3rd ovalbumin challenge, a time at which no late asthmatic response was observed. No further increase was observed before and 4 h after the 4th challenge (Fig. 8D). Together, these data suggest no correlation between the numbers of lung IgE^+, $CD117^-$ cells and the ability to express a late asthmatic response. The increases of both the IgE^+, $CD117^+$ cells and the IgE^+, $CD117^-$ cells were strongly suppressed by treatment with dexamethasone using the successive dosing regimen (Fig. 8E and F).

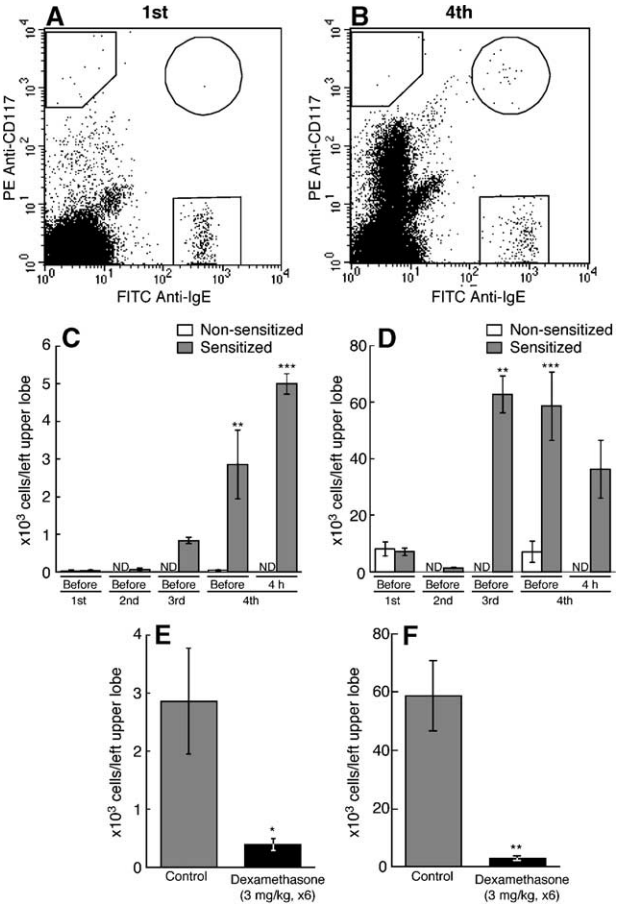

Fig. 8. Numbers of IgE^+, $CD117^+$ cells and IgE^+, $CD117^-$ cells recovered in suspensions of cells from lungs are increased after successive antigen challenge. (A and B) Typical flow cytometric profiles of IgE^+, $CD117^+$ cells and IgE^+, $CD117^-$ cells in cell suspensions from the lungs of sensitized mice isolated before the 1st (A) and the 4th (B) i.t. ovalbumin challenges. (C and D) Time-course of changes in numbers of IgE^+, $CD117^+$ cells (C) and IgE^+, $CD117^-$ cells (D) isolated from lungs of non-sensitized and sensitized mice before the 1st, 2nd, 3rd and 4th challenges and 4 h after the 4th challenge. Each data point represents the mean±S.E.M. of 4–6 animals. ** and ***: $P<0.01$ and 0.001, respectively, vs. values before the 1st challenge. (E and F) Effect of successive dosing administration of dexamethasone on increases of IgE^+, $CD117^+$ cells (E) and IgE^+, $CD117^-$ cells (F) in cell suspensions of lungs isolated from the sensitized mice before the 4th challenge. Each bar represents the mean number of cells±S.E.M. of 5 or 6 animals. Dexamethasone (3 mg/kg/dose) was administered i.p. 2 h before the 1st, 2nd, 3rd and 4th challenges and once a day on each of the 2 days between the 3rd and 4th challenges. * and **: $P<0.05$ and 0.01, respectively, vs. control. ND, not determined.

4. Discussion

In order to assure that a large fraction of the antigen reached the lower airway and to reduce allergic responses in the nasal tissues as much as possible, we chose to administer the challenge antigen intratracheally. Using this challenge protocol, we found that in mice that had been sensitized systemically with ovalbumin and conditioned by intratracheal ovalbumin challenges on three successive days, the late asthmatic response could be reproducibly and clearly induced by a 4th i.t. ovalbumin challenge. In previous studies of the late asthmatic response in mice (Cieslewicz et al., 1999; Karras et al., 2000), antigen challenge had been performed using inhalation of the antigen as an aerosol mist. In prior studies using guinea pigs, we included Evans blue dye in the nebulized antigen solution in order to assess the fraction of the total mist that was localized in the upper airway (specifically in the nasal cavity). In that study, we found that 80% of the Evans blue was retained in the upper airway (Nabe et al., 1997b). We anticipate that the intratracheal antigen challenge used in the experiments reported here delivers the highest possible fraction of the challenge antigen to the lower airways. We expect, consequently, that this form of challenge more effectively elicits allergic responses in the lungs, enhancing the value of this model for analysis of asthma.

A key finding in our current study is that the late asthmatic response was not induced by the 1st, 2nd or 3rd i.t. challenges of sensitized mice, but required a 4th challenge that was administered 3 d after the third. Our data suggest that the first three airway antigen challenges elicited changes that conditioned the mice for a qualitatively different response as a consequence of the 4th challenge. Although the first three airway challenges clearly were capable of eliciting systemic immune responses (manifested, for example, as increased levels of serum IgE and IgG1 anti-ovalbumin antibodies), we anticipated that the responses that permitted the subsequent development of an antigen-induced late asthmatic response were local ones expressed in the lungs. Consequently, we carefully examined the pattern of leukocyte infiltration into the lungs and airways over time following each of the i.t. antigen challenges. Studies using microscopic evaluation of lung sections and flow cytometric and cytospin evaluation of BAL fluid showed that repeated antigen challenges induced recruitment of a broad range of leukocytes, including neutrophils, eosinophils, $CD4^+$ T cells, $CD8^+$ T cells, and other mononuclear cells. The similarity in neutrophil infiltration patterns between non-sensitized and sensitized mice indicated that airway challenge-induced neutrophil recruitment is an antigen non-specific response, probably driven by innate aspects of the immune response. In contrast, recruitment of $CD4^+$ T cells, $CD8^+$ T cells, and eosinophils was dependent on prior antigen sensitization. Importantly, while there had been little cell accumulation prior to the 1st, 2nd and 3rd challenges, infiltration with all of these cell types had been markedly induced by the time of the 4th challenge. Recently, it has been shown that systemic treatment with neutralizing antibodies or with other types of inhibitors of the adhesion molecules, very late activation antigen-4 (Sagara et al., 1997; Abraham et al., 2000) or L-selectin (Abraham et al., 1999), suppressed late asthmatic responses in sheep and guinea pigs. Thus, we anticipate that further studies investigating the roles of adhesion molecules in the leukocyte infiltration observed in our model should be helpful for elucidating the relationship between the leukocyte recruitment and the induction of the late asthmatic response.

In addition to finding increased numbers of leukocytes in the BAL fluid harvested after the 3rd and 4th challenges, we found that the numbers of IgE^+, $CD117^+$ cells (mast cells as suggested by Yamaguchi et al. (1997)) were also increased in single cell suspensions of dissociated lung tissue isolated at these times. It is well established that mast cells are present in increased numbers in the lungs of asthmatic patients (Tomioka et al., 1984; Flint et al., 1985; Kirby et al., 1987); however, few studies have noted lung mast cell hyperplasia in murine models of allergic asthma. Perhaps complicating such analyses is our observation of relatively large number of IgE^+, $CD117^-$ cells in the lungs of sensitized, allergen challenged mice. Previous studies (Luccioli et al., 2002; Novak et al., 2003) have shown that these cells consist of basophils and a subset of antigen presenting cells. In our study, we found that these IgE^+, $CD117^-$ cells were increased even by the 3rd antigen challenge at a time when no late asthmatic response was observed. This indicates that these IgE^+, $CD117^-$ cells are not sufficient to drive the late asthmatic response.

Altogether, our studies demonstrate a parallel accumulation of $CD4^+$ and $CD8^+$ T cells, eosinophils, and IgE^+, $CD117^+$ cells, and demonstrate a correlation between this airway-associated inflammation and the ability to manifest a late asthmatic response following i.t. antigen challenge. We suggest that these inflammatory changes are causally related to the ability of antigen to induce the late asthmatic response following the 4th challenge. In contrast to other reports using different challenge schedules (Cieslewicz et al., 1999; Tomkinson et al., 2001), we observed that the 4th antigen challenge, although it induced a clear late asthmatic response, caused little further increase in recruitment of leukocytes compared to the recruitment induced by the first 3 challenges. One explanation for this result is that our antigen challenge schedule may elicit maximal lung inflammation by the time the 4th challenge is administered.

Supporting the hypothesis that the first 3 airway antigen challenges culminate in the induction of sufficient airway inflammation to support the subsequent late asthmatic response are our data showing that the production of Th2-type cytokines as detected in BAL fluid is progressively induced over the course of the 1st–4th challenges. Increases of the Th2-type cytokines IL-4, IL-5 and IL-13 were clearly detected in BAL fluid collected during the sequential antigen challenges. Interestingly, the Th1-type cytokine IFN-γ was not detected at any time point using this challenge protocol. Based on these cytokine profiles, we assert that the induction of the late asthmatic response is related to Th2-type lung inflammation. Interestingly, the time course patterns that characterized the production of the individual Th2-type cytokines were different from each other, suggesting either that the cellular sources of the individual cytokines were not identical or that different factors govern the production of each effector protein. In contrast to the production patterns of the purely Th2-type cytokines, the production pattern of IL-10 was unique because it was increased only after the 4th challenge. IL-10 has been known to be an anti-inflammatory cytokine, and produced by some populations of regulatory T cells (Hawrylowicz and O'Garra, 2005). It is possible that T cells capable of producing IL-10 accumulate in the lungs prior to the 4th challenge, but are not activated to secrete the cytokine until they are triggered by the 4th challenge. Although the pathophysiological actions of IL-10 during the late asthmatic response are not fully defined, it is possible that initiation of the tissue recovery process may overlap the induction of the late asthmatic response. Alternatively, IL-10 may play an active role in the induction of the late asthmatic response. Involvement of IL-10 in the induction of airway hyperresponsiveness in mice has been reported in studies using IL-10 deficient mice (Mäkelä et al., 2000) and in mice treated with IL-10 in association with airway antigen challenge (van Scott et al., 2000). Additional studies will be required to elucidate the roles and cellular sources of IL-10 produced during the late asthmatic response in our model.

In contrast to the levels of Th2-type cytokines, the levels of both ovalbumin-specific IgE and IgG1 antibodies in the sera of sensitized mice were already high before the 1st challenge. IgE levels but not IgG1 levels were reduced following each of the first two challenges. This decrease may have indicated consumption of IgE by the potent anaphylactic reaction that was manifested by the marked early asthmatic response after the 1st challenge. Crosby et al. (2002) reported that the early asthmatic response in C57BL/6J mice required allergen-specific IgG antibody but not IgE or mast cells. Although our experiments do not directly demonstrate a dependence on IgE antibody and mast cells for the induction of the early asthmatic response, the consumption of IgE antibody supports the speculation that mast cells and IgE play important roles in the induction of early asthmatic response at least for the 1st antigen challenge. The reduced IgE levels observed after the 1st challenge correlate with the loss of the early asthmatic response at the 2nd and 3rd challenges. In addition, the absence of the early asthmatic response at the 2nd and 3rd challenges may be linked to depletion of mast cell granule content following the robust anaphylaxis at the 1st challenge.

By the time of the 4th challenge, ovalbumin-specific IgE levels were restored to those observed prior to the 1st challenge. Furthermore, ovalbumin-specific IgG1 antibody levels were also increased at the time of the 4th challenge compared to the levels that were detected at the times of the 1st, 2nd, and 3rd challenges. Although these data showed a correlation between elevated antigen-specific IgE and IgG1 level and the ability to induce a late asthmatic response, daily treatment with dexamethasone demonstrated that this increase in antigen-specific immunoglobulin was not sufficient to cause the late asthmatic response. Daily treatment with dexamethasone potently suppressed the 4th challenge-induced early asthmatic response, but the levels of neither IgE nor IgG1 antibodies were reduced by the corticosteroid. In addition to their ability to up-regulate circulating levels of ovalbumin-specific IgE and IgG1, repeated airway antigen challenges also increased the numbers of lung FcεRI-positive mast cells (IgE^+, $CD117^+$) and basophils (IgE^+, $CD117^-$ cells). These results suggest that the mechanisms that induce the early asthmatic responses at the times of the 1st and the 4th antigen challenges may be different from each other. The increased numbers of mast cells and basophils that are present at the time of the 4th antigen challenge may contribute importantly to the induction of the early asthmatic response induced by intratracheal antigen at that time.

Unlike the early asthmatic response, the late asthmatic response is thought to depend on acute triggers that follow the development of airway inflammation. In the present study, both leukocyte infiltration into the lungs and mucus accumulation (increased numbers of alcian blue/periodic acid Schiff-positive cells) were observed at the airway epithelia immediately prior to the 4th antigen challenge. This challenge induced the late asthmatic response. In contrast, very little airway inflammation and no mucus hyperplasia were observed prior to the 2nd and 3rd i.t. ovalbumin challenges, challenges that yielded no late asthmatic response (unpublished data). Consistent with these observations based on analysis of BAL fluid and of histological sections of lung tissue, we could detect wheezing around 2–6 h after the 4th i.t. antigen challenge but not after the 1st–3rd challenges of sensitized mice. We suggest the hypothesis that these changes in airway cellularity and mucus production are manifestations that underlie the late phase increase in Penh and sRaw that we define as the late asthmatic response.

In order to investigate further the relationship between airway inflammation and the early and late asthmatic responses, we tested whether the antigen-induced asthmatic responses observed in this model could be suppressed by treatment with corticosteroids, a major class of therapeutic drugs frequently used for asthma therapy. Both the early and late asthmatic responses were strongly suppressed by treatment with dexamethasone when administered using the successive dosing protocol. Administration of a single large dose of dexamethasone prior to the 4th antigen challenge did not show a major effect on either the early or late asthmatic response. Consistent with its effect on pulmonary mechanics, the successive dosing, but not the single dose regimen of corticosteroid treatment significantly inhibited recruitment of leukocytes into BAL fluid, as well as production of cytokines and accumulation of mucus in the airway epithelial cells. This parallel inhibition of early and late asthmatic responses, of recruitment of inflammatory cells, and of production of Th2 type cytokines by the successive dosing but not by the single dose corticosteroid treatment regimen suggests that the mechanisms that induce the early and late asthmatic responses are fundamentally dependent on the lung inflammation that had been established by the time of the 4th challenge. In addition, the requirement for the successive dosing treatment with the corticosteroid in order to inhibit the late asthmatic response is consistent with the requirement for multiple i.t. challenges to induce the late asthmatic response.

It has been generally accepted that the early asthmatic response is induced mainly by airway smooth muscle contraction, and that corticosteroids have no direct effect on this contraction. We have demonstrated here, however, that the dexamethasone multiple dosing regimen inhibited development of both the early and late asthmatic responses. The inhibition of the early asthmatic response by this successive dosing regimen prompted us to evaluate the control of mast cell numbers in the lung tissue of antigen challenged animals. Dexamethasone almost completely suppressed the antigen-induced increases of IgE^+, $CD117^+$ cells (mast cells) and IgE^+, $CD117^-$ cells (basophils and some antigen-presenting cell subtypes) in the lung. This result was consistent with the hypothesis that the increased numbers of mast cells that had accumulated by the time of the 4th antigen challenge underlay the early and late asthmatic responses that could be induced at that time point. Interestingly, the numbers of lung IgE^+, $CD117^-$ cells (basophils and antigen-presenting cells) were dramatically increased by time of the 3rd antigen challenge, a time at which there was little early asthmatic response and no detectable late

asthmatic response after i.t. antigen challenge. This suggested that basophils were not sufficient to drive the development of these obstructive airway responses. Altogether, these data were consistent with dexamethasone inhibition of the early asthmatic response being mediated primarily by the suppression of accumulation of lung mast cells.

In conclusion, our studies have shown that: [1] multiple i.t. allergen challenges induced a reproducible late asthmatic response as well as early asthmatic response in sensitized BALB/c mice; [2] the mechanisms leading to development of the late asthmatic response correlate with establishment of lung inflammation including recruitment and activation of inflammatory cells; and [3] increased numbers of lung mast cells may play important roles in the induction of the early and the late asthmatic responses.

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