

Eosinophilic rhinitis accompanies the development of lower airway inflammation and hyper-reactivity in sensitized mice exposed to aerosolized allergen

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Summary

Background Allergic rhinitis is a risk factor for the development of asthma. About 80% of asthmatic patients also have rhinitis. However, the pattern of induction of allergic rhinitis and asthma remains unclear.

Objective The purpose of this study was to investigate the development of upper airway inflammation in mice during the development of an asthma-like disease and after an acute allergen provocation.

Methods BALB-c mice were sensitized intraperitoneally (i.p) to ovalbumin (OA, days 1–13) and were challenged with aerosols of either OA or saline on 8 consecutive days (days 33–40). In a second experiment, chronic exposure for 8 days was followed by 10 days of rest and then an acute nebulized allergen provocation was performed (day 50). Inflammatory parameters were investigated at different time-points.

Results Upper and lower eosinophilic airway inflammation were simultaneously induced in the course of repeated inhalations of nebulized OA, as shown by analyses of nasal and broncho-alveolar lavage fluids and histological sections of the nose and bronchi. Mice that developed bronchial hyper-responsiveness also had increased thickness of the nasal mucosa on magnetic resonance image (MRI) scans. When chronic exposure was followed by acute allergen provocation, the latter caused a systemic increase in IL-5 levels, with a concomitant rise in blood and airway eosinophils, primarily in the nose.

Conclusions Simultaneous induction of eosinophilic inflammation in the nose and lungs was found in a mouse model of respiratory allergy. These findings support the viewpoint that upper and lower airway disease represent a continuum of inflammation involving one common airway and provide evidence for the concept of global airway inflammation after inhalation of allergen.

Keywords: allergy, rhinitis, asthma, ovalbumin, airway challenge, allergen provocation

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Introduction

Epidemiological studies have consistently shown that allergic asthma and rhinitis often coexist in the same patients [1,2]. Both airway diseases share the same trends of increasing incidence [3], predisposing factors [2],

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pathophysiological mechanisms following allergen encounter [4,5] and beneficial effect of treatment with topical corticosteroids [6]. Some authors suggest that allergic rhinitis and asthma are manifestations of the same disease in two parts of the respiratory tract and that asthma represents the more severe end of the spectrum [4,7,8].

Recent murine models of allergic rhinitis [9–12] and all studies on asthma in mice have focused on one part of the airway in isolation without studying rhinitis in the context of asthma. The aim of the present study was to investigate the upper respiratory tract in a murine asthma model, induced by chronic aerosolized allergen exposure of previously sensitized mice. In contrast to local allergen application, as carried out in existing murine models of allergic rhinitis [9–12], mice in our study chronically inhaled nebulized allergen solution on 8 consecutive days. This allergen exposure more closely mimics allergen exposure in rhinitis patients. The upper airways were then investigated by histological examination of nasal mucosa, analysis of nasal lavage fluid (NLF) and radiological imaging of the nose. Lower airway inflammation and asthma were confirmed in these same mice by analysis of bronchial mucosa, broncho-alveolar lavage fluid (BALF) and airway hyper-responsiveness. In addition to this model of chronic exposure, we also studied the effects of acute allergen exposure, mimicking acute allergen exposure in patients with respiratory allergy. To that end, sensitized mice that had chronically inhaled allergen were exposed to an aerosol containing OA after an allergen-free interval of 10 days. The acute effects of this exposure were investigated.

A murine model of total allergic airway disease is presented that adds weight to the argument that rhinitis is ubiquitous in allergic asthma and also creates a new tool for research on upper allergic airway disease.

Materials and methods

Experimental protocol

Male BALB-c mice (6 weeks of age, Harlan, Horst, the Netherlands) were kept under conventional pathogen-free conditions, including cages with filter-cap, irradiated food and water, and manipulations under laminar air-flow. Experimental procedures were approved by the local Ethical Committee of Animal Experiments. Mice were actively sensitized by seven intraperitoneal injections of 10 µg OA (grade V, Sigma, St Louis, MO, USA) in 0.5 mL of pyrogen-free saline on alternate days from day 1 till day 13. From day 33 till day 40, mice inhaled nebulized OA (10 mg/mL, OA group) or saline (saline group) daily for 5 min, generated by a jet-nebulizer (PARI TurboBOY, Starnberg, Germany) connected to a plexiglass exposure chamber (5.7 L). To study the effects

of chronic allergen exposure, airway inflammation was evaluated in groups of six OA mice on days 34, 37 and 41, which is 24 h after the first, fourth and eighth aerosols, respectively. The results were compared with those of six mice from the saline group. As saline challenge did not affect any parameter, only the results of saline-group mice sacrificed on day 41 are presented and compared with OA mice.

In a second part of the study, OA sensitized (days 1–13) and OA challenged (days 33–40) mice were exposed to an acute provocation with nebulized OA (10 mg/mL for 5 min) on day 50. Nasal and bronchial lavages and histological examinations of the nose and lungs were performed in groups of six mice before and at 15 min and 1, 3, 6, 12, 24, 48 and 72 h after this acute allergen provocation.

Nasal and broncho-alveolar lavages

Mice were anaesthetized by intraperitoneal (i.p.) injection of urethane (2.1 g/kg) and their four limbs and cheeks were tightly attached to the operation table in a supine position with the neck hyper-extended. A polyethylene catheter (Ø 0.85 mm) connected to a syringe was gently inserted into each nostril. One mL of phosphate-buffered saline (PBS) supplemented with 5% of bovine serum albumin (BSA, Sigma) at 37 °C was slowly injected into one nasal cavity while this fluid was simultaneously aspirated via the catheter in the contralateral nostril. In this way, both nasal cavities were flushed and on average 0.7 mL of lavage fluid was obtained. The NLF was centrifuged (4000 g, 5 min) and the supernatant stored at –20 °C until analysis. The pellet was resuspended in 100 µL of PBS for cell counting. Then the lungs were lavaged five times with 1-mL aliquots of pyrogen-free PBS at 37 °C through a polyethylene tracheal catheter (Ø 0.85 mm). The first lavage was performed with 1 mL PBS supplemented with 5% BSA, centrifuged (4000 g, 5 min) and the supernatant stored at –20 °C until analysis. The cellular pellet was added to the subsequent four lavages, each one performed with 1 mL of PBS.

The BALF pool was centrifuged (4000 g, 5 min) and the pellet washed and resuspended in 100 µL of PBS. Ten µL of cell suspension from NLF and BALF were added to 90 µL of Türk's solution (Merck Diagnostica, Darmstadt, Germany) and the total number of cells counted in a Bürker-Türk chamber. For differential cell counts, 10×10^5 cells were spun (1400 g, 6 min, Cytospin 3, Shandon, TechGen, Zellik, Belgium) upon microscope slides, air-dried and stained according to the classic May-Grünwald-Giemsa method. Two hundred cells were differentiated into eosinophils, neutrophils, monocytes and lymphocytes.

Histology

The murine respiratory tract was removed for histological examination after nasal and broncho-alveolar lavages. Skulls were skinned, fixed for 48 h in buffered formalin (5%), and decalcified during 2 weeks using a 14% ethylenediaminetetraacetic acid (Sigma) solution. Coronal

sections of the skulls at the middle third between nose-tip and orbit were made and stored in formalin until further processing. The lungs were dissected and fixed overnight in buffered formalin (5%). After dehydration and embedding in paraffin, 5- μ m sections were stained with haematoxylin and eosin (H & E). Eosinophils could be recognized on microscopic sections by their polyglobular nucleus and bright cytoplasmic granules (Fig. 1b). Representative nasal sections were scored by counting eosinophils in (sub-)epithelial layers of both lateral nasal walls between the middle and inferior nasal turbinates. In the lung, the maximal thickness of the inflammatory infiltrate around bronchioli and arterioli in the left and right lung was measured using an eyepiece graticule at a magnification of 25, and the mean of these four values was calculated.

Magnetic Resonance Image of the nose

After anaesthesia as above, coronal magnetic resonance image (MRI) scans of the mice were made on day 41 in an experimental setting that was designed for making high resolution (3×10^{-2} mm) scans of murine tissues. We used a 30-cm bore horizontal super-conducting 4.7-T magnet (Bruker BIOPSEC) equipped with a microscopy gradient insert with a 6-cm bore. T1-weighted coronal images were acquired using a spin-echo sequence (TR/TE = 1000/15 ms), with field of view of 10 mm, matrix of 256×256 , slice thickness of 1 mm and 16 acquisitions. The mice were positioned in such a way that the nose fitted under a horizontal transmit/receive surface coil with a diameter of 17 mm.

ELISA for OA-specific IgE

After anaesthesia as above, a retro-orbital bleed was performed on days 0, 28 and 41, and the serum frozen until analysis. Nunc-Immuno 96-well plates (Life Technologies, Rockville, MD, USA) were coated overnight with

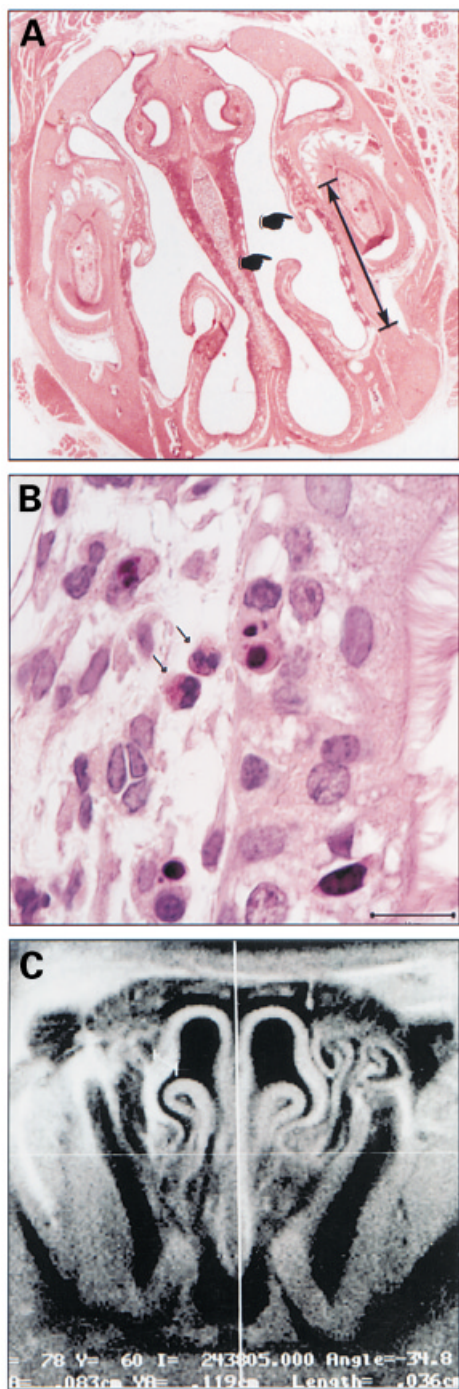


Fig. 1. Microscopic and radiological images of the murine nose. (a) Light microscopic image (10-fold magnification) of a coronal section through the sinonasal skeleton showing the murine nasal anatomy. (b) A representative image (magnification of 1500, Axioplan microscope, Zeiss) of the nasal epithelium and subepithelial layer of a sensitized and repeatedly challenged (day 33–40) mouse sacrificed on day 41. For quantification of eosinophilic inflammation, eosinophils (black arrows, b) were counted on H & E stained sections in a defined region of the nasal mucosa (along black line, a) between the lower edge of the middle turbinate (upper index, a) and the upper edge of the lower turbinate (lower index, a). (c) Coronal MRI scan of the murine skull on which measurements of thickness of nasal mucosa were performed at the lateral superior nasal wall (white line) using an interactive computerized program.

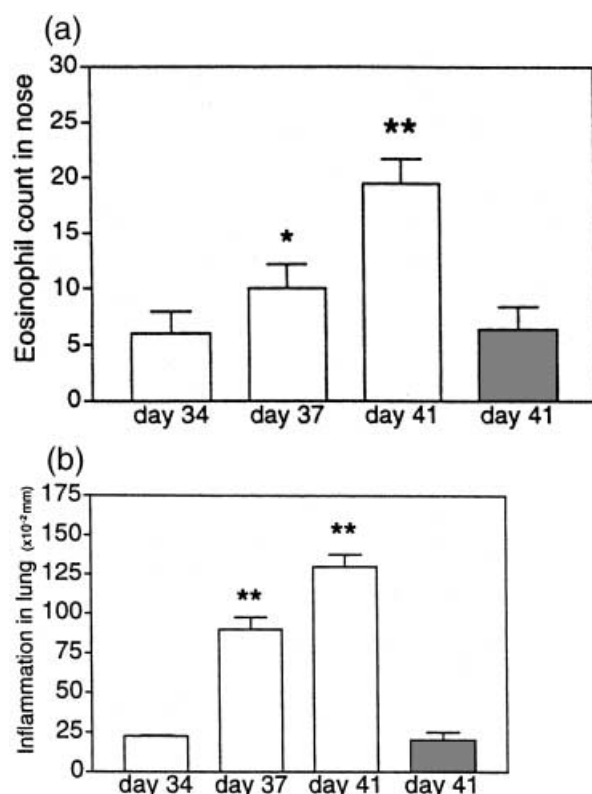


Fig. 2. Histological analyses of upper and lower airway mucosa during repeated challenges with either OA or saline of previously sensitized mice. (a) Mean eosinophil counts ($\pm$ SE) in nasal mucosa between the lower edge of the middle turbinate and the upper edge of the lower turbinate (cfr. Fig. 1a). (b) Mean thickness ($\pm$ SE) of (peri-)bronchiolar inflammation in OA-sensitized mice after one (day 34), four (day 37) and eight (day 41) aerosols with OA (open bars) or eight aerosols with saline (filled bars). * $P < 0.05$ and ** $P < 0.005$ compared with saline-challenged mice (Mann–Whitney test); each group consisted of five–six mice.

monoclonal rat anti-mouse IgE (10 μ g/mL, Pharmingen, San Diego, CA, USA), blocked with 2% BSA and incubated for 2 h with diluted (1 : 5) serum samples. After washing, OA (1 μ g/100 μ L) was added for 1 h while shaking and washed away. Peroxidase-labelled rabbit anti-OA IgG (2.4 μ g/mL, Rockland, Gilbertsville, PA, USA) was then added for 1 h while shaking. After washing, the substrate tetramethylbenzidine dihydrochloridhydrate (Acros Organics, NJ, USA) and H₂O₂ were added in buffer. The reaction was stopped by adding H₂SO₄ and the optical density measured at 450 nm. Prior to these experiments, a reference pool of serum of OA-sensitized BALB-c mice was made and its level of OA-specific IgE was arbitrarily assigned a value of 100 EU/mL. IgE values of other mice were expressed in percentages of the reference pool.

ELISA for IL-5

IL-5 levels in undiluted NLF and serum and 1 : 2 diluted BALF were measured according to the manufacturer's instructions (Pharmingen), with the use of rat anti-mouse IL-5 and rat anti-mouse IL-5 conjugated with biotin. The lower limit of detection was 4.5 pg/mL.

Measurement of airway responsiveness

Airway responsiveness was assessed by metacholine-induced airflow obstruction in conscious mice placed in a whole body plethysmograph (Buxco®, EMKA Technologies, Paris, France) as described by Hamelmann *et al.* [13]. Airway responsiveness was evaluated by the enhanced pause (Penh), a calculated value which correlates with measurement of airway resistance [13]. $\text{Penh} = (\text{Te}/\text{Tr} - 1) \times (\text{Pef}/\text{Pif})$. (Te = expiration time; Tr = relaxation time; Pef = peak expiratory flow; Pif = peak inspiratory flow.) The Penh was calculated for each mouse under resting conditions and during 3 min after nebulizing physiological saline and incremental doses of metacholine (10, 20 and 50 mg/mL) for 1 min. The mean Penh value of each mouse was calculated during 1 min at rest and at the time of maximal response after each aerosol.

Data analyses

Data are expressed as means $\pm$ SEM. Statistical analyses were carried out using the Mann–Whitney test. A difference was considered to be significant when $P < 0.05$. A Spearman correlation test was performed for correlations between analyses of lavage fluids and histology.

Results

Induction of IgE and lower airway hyperresponsiveness

Sensitization of mice by i.p. injection of OA on alternate days from day 1 till day 13 induced production of OA-specific IgE antibodies. On day 28, serum levels of OA-specific IgE were 91.5 ± 13.4 EU/mL, compared to 0.5 ± 0.3 EU/mL ($P < 0.0001$) before sensitization. Repeated exposure of sensitized mice to nebulized OA (OA mice) for 8 consecutive days caused a further but not significant ($P = 0.218$) increase in OA-specific IgE levels (109.0 ± 8.7 EU/mL) and induced airway hyperresponsiveness. Penh values of OA mice ($n = 6$) rose to higher levels than saline mice ($n = 6$) after inhalation of nebulized metacholine at 10 mg/mL (2.4 ± 0.8 vs. 1.2 ± 0.4 ; $P < 0.005$), 20 mg/mL (3.4 ± 0.9 vs. 2.1 ± 0.7 ; $P = 0.005$) and 50 mg/mL (9.2 ± 3.1 vs. 3.7 ± 1.2 ; $P < 0.0001$). Penh values were not different between both

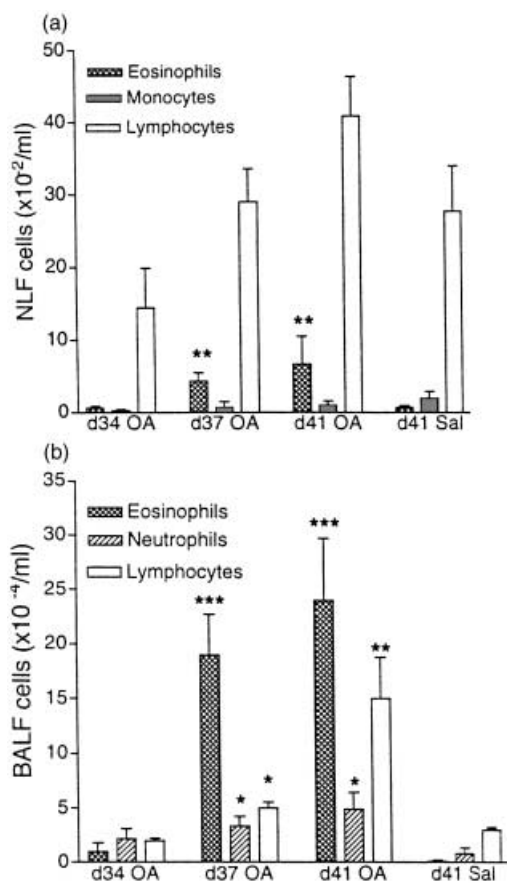


Fig. 3. Lavage fluid analyses of upper and lower airways during repeated challenges with either OA or saline of previously sensitized mice. Differential cell counts in NLF (a) and BALF (b) of OA-sensitized mice sacrificed 24 h after one (day 34), four (day 37) and eight (day 41) challenges with nebulized OA, were compared with cell counts of OA sensitized mice sacrificed after eight challenges (day 41) with nebulized saline. * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.0001$ compared with saline-challenged mice; each group consisted of five–six mice.

groups at rest (0.39 ± 0.1 and 0.37 ± 0.2 , respectively) and after inhalation of nebulized physiological saline (0.40 ± 0.4 and 0.36 ± 0.3 , respectively).

Repeated exposure to aerosolized allergen induces nasal eosinophilic inflammation in parallel with lower airway inflammation

The murine nose shows many similarities to human anatomy, including nasal septum, turbinates and paranasal sinuses (Fig. 1a). Airway inflammation in the nose and lungs was studied histologically after one, four and eight daily airway challenges with OA or saline. The nasal inflammatory infiltrate in OA mice was present mainly in the

subepithelial layer along the lower part of the nasal septum and lateral nasal wall, and consisted primarily of eosinophils and mononuclear cells (Fig. 1b). Eosinophil infiltration was quantified by counting eosinophils in the mucosa between the lower edge of the middle turbinate and the superior edge of the lower turbinate (Fig. 1a). As shown in Fig. 2(a), eosinophil counts in the defined nasal region ranged from 6.0 ± 1.9 to 10.1 ± 2.2 and 19.4 ± 2.2 after one, four and eight OA challenges (i.e. on days 34, 37 and 41, respectively). On days 37 and 41, nasal eosinophil counts in OA mice were higher than in saline mice on day 41 (6.3 ± 2.0 , $P < 0.05$ and 0.005 , respectively, Fig. 2a). In the lung, the mean thickness of perivascular and peribronchiolar inflammation increased from $22.5 \pm 0.5 \times 10^{-2}$ to $90.0 \pm 7.5 \times 10^{-2}$ and $130.0 \pm 7.5 \times 10^{-2}$ mm after one, four and eight challenges with OA, respectively (Fig. 2b). Also in lung tissue, OA mice on days 37 and 41 showed more inflammation than saline mice ($20.0 \pm 5.0 \times 10^{-2}$ mm, $P < 0.005$, Fig. 2a).

Magnetic resonance imaging scans reveal thickening of nasal mucosa after repeated allergen challenge of sensitized mice

To study nasal mucosal changes *in vivo*, a MRI study of the nose and paranasal sinuses was performed on day 41, which is 24 h after the last aerosol with saline ($n = 5$) or OA ($n = 5$). The mucosal thickness (Fig. 1c) at the level of the superior lateral nasal wall was measured with the aid of a computerized processing program of magnified MRI scans. This region was chosen for analysis because of the presence of eosinophilic inflammation on histology and the clear visualization of this mucosa on MRI scans. Measurements of mucosal thickness were performed on each nasal side and on two scans of each mouse. The mean mucosal thickness was $27.4 \pm 1.6 \times 10^{-2}$ mm in OA mice and $19.1 \pm 1.2 \times 10^{-2}$ mm in saline mice, which represented a significant ($P = 0.008$) increase of 42% in OA mice. The mucosal swelling in OA mice was allergen-specific as pilot studies showed that eight daily inhalations of nebulized OA solution (10 mg/mL) by sham-sensitized mice (mean: 20.1×10^{-2} mm, $n = 2$) or inhalations of nebulized BSA solution (10 mg/mL) by OA sensitized mice (mean: 19.2×10^{-2} mm, $n = 2$) did not cause any mucosal swelling.

Cellular composition of nasal and bronchial lavage fluid during chronic allergen exposure

In NLF of OA mice, eosinophil counts increased from $0.7 \pm 0.2 \times 10^2$ to $4.4 \pm 1.2 \times 10^2$ and $6.7 \pm 3.8 \times 10^2$ mL after one (day 34), four (day 37) and eight (day 41) daily challenges with aerosolized OA, respectively

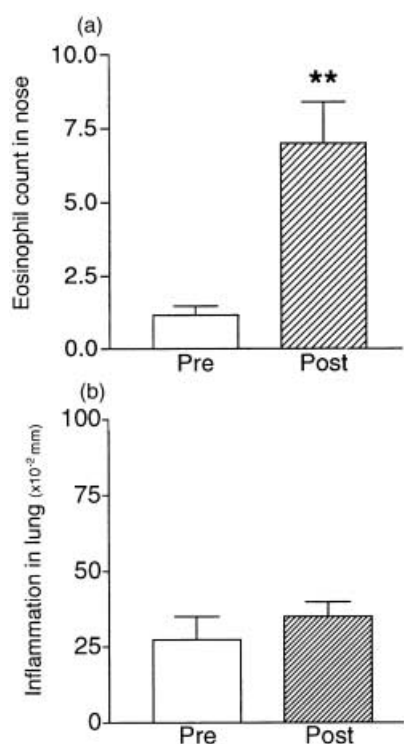


Fig. 4. Histological analyses of upper and lower airway inflammation before and after acute allergen provocation. After sensitization (days 1–13) and chronic aerosolized allergen exposure (days 33–40), an allergen provocation was carried out on day 50 and the results of mice analysed before (pre) and at 6 h after (post) provocation are presented. Eosinophil counts in the defined region of the nose (a, cfr. Fig. 1a) and thickness of (peri)bronchiolar inflammation (b) were compared between pre and post mice. ** $P < 0.005$; each group consisted of five–six mice.

(Fig. 3a). Higher eosinophil counts were found in OA mice on days 37 and 41 compared with saline mice on day 41 ($0.7 \pm 0.3 \times 10^2/\text{mL}$, $P < 0.005$).

Lymphocyte numbers increased from $14.5 \pm 5.3 \times 10^2$ to $29.0 \pm 4.6 \times 10^2$ and $40.9 \pm 10.8 \times 10^2/\text{mL}$ in OA mice on days 34, 37 and 41, respectively, but the difference with the control was not significant ($27.8 \pm 6.2 \times 10^2/\text{mL}$, $P = 0.393$, Fig. 3a). Monocyte counts did not differ among the four groups of mice analysed: $0.8 \pm 0.2 \times 10^2$, $0.8 \pm 0.5 \times 10^2$, $1.0 \pm 0.7 \times 10^2$ and $2.0 \pm 1.0 \times 10^2/\text{mL}$ for OA mice on days 34, 37 and 41 and saline mice on day 41, respectively. Neutrophil counts in NLF (not illustrated) constituted the most frequent cell type (mean: 75%) and their number did not differ among the groups analysed: $6.7 \pm 0.6 \times 10^3$, $6.0 \pm 1.1 \times 10^3$ and $4.8 \pm 0.9 \times 10^3/\text{mL}$ in OA mice on days 34, 37 and 41, respectively, and $6.9 \pm 0.6 \times 10^3/\text{mL}$ in saline mice on day 41. Total cell counts in NLF were similar in all groups: $7.5 \pm 0.4 \times 10^3$, $9.4 \pm 0.9 \times 10^3$ and $9.7 \pm 0.8 \times 10^3/\text{mL}$

in OA mice on days 34, 37 and 41, and $9.9 \pm 0.7 \times 10^3/\text{mL}$ in saline mice on day 41, respectively.

In BALF, the number of eosinophils increased progressively from $1.0 \pm 0.8 \times 10^4$ to $19.1 \pm 4.7 \times 10^4$ and $24.0 \pm 5.8 \times 10^4/\text{mL}$ in OA mice on days 34, 37 and 41, respectively (Fig. 3b). Eosinophil counts in BALF of OA mice on days 37 and 41 were higher than in saline mice ($0.1 \pm 0.1 \times 10^4/\text{mL}$, $P < 0.0001$). Neutrophil counts in BALF of OA mice ranged from $2.1 \pm 0.9 \times 10^4$ to $3.3 \pm 0.9 \times 10^4$ and $4.8 \pm 1.5 \times 10^4/\text{mL}$ on days 34, 37 and 41, respectively. Also neutrophil counts were higher in BALF of OA mice on days 37 and 41 than in BALF of saline mice ($0.8 \pm 0.5 \times 10^4/\text{mL}$, $P < 0.05$, Fig. 3b). Lymphocyte counts increased from $2.1 \pm 0.2 \times 10^4$ to $5.0 \pm 0.5 \times 10^4$ and $15.0 \pm 3.8 \times 10^4/\text{mL}$ in BALF of OA mice on days 34, 37 and 41, respectively, and counts in the two latter mice outnumbered those in saline mice ($3.0 \pm 0.2 \times 10^4/\text{mL}$, $P < 0.05$ and 0.005 , respectively, Fig. 3b). Monocytes (not illustrated) were the most frequent cell type in BALF of saline mice ($3.7 \pm 1.3 \times 10^5/\text{mL}$) and their number did not change upon challenge with OA: $4.0 \pm 0.3 \times 10^5$, $3.8 \pm 1.2 \times 10^5$ and $2.8 \pm 0.5 \times 10^5/\text{mL}$ in OA mice on days 34, 37 and 41, respectively. Neither did the total number of cells in BALF differ among the groups of mice analysed: $4.5 \pm 1.6 \times 10^5$, $6.6 \pm 1.9 \times 10^5$ and $7.2 \pm 2.3 \times 10^5/\text{mL}$ after one, four and eight OA challenges, respectively, and $4.0 \pm 1.2 \times 10^5/\text{mL}$ after eight saline exposures.

Effect of acute allergen exposure

To mimic the situation in which a patient with chronic airway allergy is acutely exposed to allergen, OA-sensitized (days 1–13) and chronically-exposed (days 33–40) mice inhaled nebulized OA (10 mg/mL) during 5 min on day 50. An allergen-free interval of 10 days (from day 40 till day 50) was chosen because inflammatory cells in BAL fluid and bronchial tissue had almost completely disappeared by day 50. Acute allergen exposure resulted in a systemic increase in IL-5 and eosinophils as evidenced by blood analysis. The level of IL-5 was $9.2 \pm 2.1 \text{ pg/mL}$ of serum before provocation and increased significantly at 6 and 12 h after provocation (81.1 ± 0.4 and $35.1 \pm 8.5 \text{ pg/mL}$, respectively, $P < 0.05$). No significant increase was recorded at any other time-point after provocation (15 min, 1, 3, 24, 48 and 72 h). The percentage of eosinophils in blood was 4.3 ± 0.4 before provocation and transiently increased with a peak at 6 h (13.0 ± 2.5 , $P = 0.041$) after OA provocation.

Airways were analysed at several time-points after provocation, i.e. 15 min, 1, 3, 6, 12, 24, 48 and 72 h. However, only the data of mice sacrificed at 6 h after provocation (post-mice) are presented because nasal

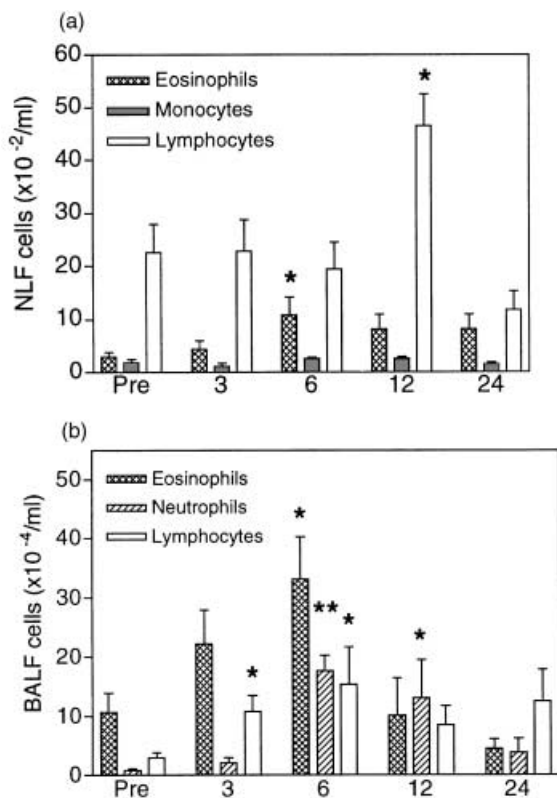


Fig. 5. Lavage fluid analyses of upper and lower airways before and after acute allergen provocation. Differential cell counts in NLF (a) and BALF (b) of mice before (pre) provocation were compared with cell counts in mice at 3, 6, 12 and 24 h after acute OA provocation. * $P < 0.05$ and ** $P < 0.005$ compared with pre mice; each group consisted of five–six mice.

inflammation was maximal at that time-point. Data of post-mice were compared with mice sacrificed on the same day before provocation (pre-mice). In the defined region of the nose, an eosinophil count of 7.0 ± 1.4 was found at 6 h after provocation (post, Fig. 4a), which represented a significant increase compared to mice analysed before provocation (pre, 1.1 ± 0.3 , $P < 0.005$). The thickness of peribronchial inflammation did not change at 6 h (Fig. 4b), nor at any other time-point after provocation.

Cellular composition and IL-5 in NLF and BALF after acute allergen provocation

The results of mice sacrificed at 3, 6, 12 and 24 h after provocation are compared with mice analysed before provocation (pre-mice). The results of mice analysed at 15 min, 1, 48 and 72 h after provocation are omitted because of the lack of any increased cell count in lavage fluids compared with pre-mice.

In NLF, an eosinophil count of $3.0 \pm 0.8 \times 10^2/\text{mL}$

before provocation increased to a maximum of $10.8 \pm 3.4 \times 10^2/\text{mL}$ ($P < 0.05$) at 6 h after provocation (Fig. 5a). Lymphocyte numbers were maximal at 12 h ($46.4 \pm 6.1 \times 10^2/\text{mL}$) after provocation, which was significantly higher than before provocation ($22.6 \pm 5.2 \times 10^2/\text{mL}$, $P < 0.05$). Monocyte numbers in NLF were not influenced by provocation. Neutrophil counts in NLF (not illustrated) did not differ among the groups: $56.7 \pm 7.1 \times 10^3$, $62.0 \pm 8.6 \times 10^3$, $47.3 \pm 6.6 \times 10^3$, $34.8 \pm 10.5 \times 10^3$ and $14.8 \pm 3.8 \times 10^3/\text{mL}$ for mice analysed before and at 3, 6, 12 and 24 h after provocation, respectively. Total cell counts in NLF were $59.4 \pm 6.7 \times 10^3/\text{mL}$ before provocation and their counts did not change upon provocation. The level of IL-5 in NLF of mice analysed at 6 h after allergen provocation was $18.8 \pm 2.1 \text{ pg/mL}$, whereas no IL-5 (lower limit of detection: 4.5 pg/mL) could be detected in NLF of mice at any other time-point after provocation.

In BALF, eosinophils reached their maximal number ($33.1 \pm 7.3 \times 10^4/\text{mL}$) at 6 h after provocation, up from $10.7 \pm 3.2 \times 10^4/\text{mL}$ before provocation ($P < 0.05$, Fig. 5b). The neutrophils in BALF had significantly increased at 6 h ($17.6 \pm 2.6 \times 10^4/\text{mL}$, $P < 0.005$) and 12 h ($13.1 \pm 6.5 \times 10^4/\text{mL}$, $P < 0.05$) after provocation, in comparison to mice analysed before provocation ($0.8 \pm 0.3 \times 10^4/\text{mL}$, Fig. 5b). Lymphocyte counts were $3.0 \pm 0.9 \times 10^4/\text{mL}$ before and increased to $10.8 \pm 2.7 \times 10^4$ and $15.3 \pm 6.3 \times 10^4$ at 3 and 6 h after provocation, respectively ($P < 0.05$, Fig. 5b). Monocytes were the most numerous cell type in BALF (not illustrated) and their counts did not differ among the groups: $8.7 \pm 2.3 \times 10^5$, $13.0 \pm 2.5 \times 10^5$, $12.1 \pm 2.3 \times 10^5$, $23.1 \pm 6.5 \times 10^5$ and $10.0 \pm 17.1 \times 10^5/\text{mL}$ in mice analysed before and at 3, 6, 12 and 24 h after provocation, respectively. Total cell counts in BALF were $10.2 \pm 2.4 \times 10^5/\text{mL}$ before provocation and did not change upon provocation. IL-5 levels in BALF increased after OA provocation at 3 h ($20.9 \pm 3.4 \text{ pg/mL}$) and 12 h ($29.3 \pm 4.4 \text{ pg/mL}$) in comparison to the level before provocation ($11.7 \pm 3.2 \text{ pg/mL}$, $P < 0.05$).

Comparison of parameters of eosinophilic inflammation

Positive correlations were found between eosinophil counts in BALF and inflammatory scores of lung tissue in individual mice exposed to nebulized OA, i.e. OA mice on days 34, 37, 41 and 51 ($n = 22$, $r = 0.61$, $P < 0.005$). Eosinophil counts in NLF and in nasal tissue were positively correlated in these mice ($n = 19$, $r = 0.68$, $P < 0.005$). A highly positive correlation ($n = 18$, $r = 0.81$, $P < 0.0001$) was also observed between eosinophil counts in NLF and BALF in OA-exposed mice.

Discussion

In this study on nasal allergy we have used a model in which mice were actively sensitized with allergen in the absence of adjuvants, and then repeatedly (eight times) exposed to nebulized allergen. These mice develop an airway disease with features reminiscent of human allergic asthma [14,15]: allergen-specific IgE production, blood eosinophilia, airway hyper-responsiveness and peribronchial eosinophilic inflammation. The experimental protocol of repeated allergen exposure not only mimics the chronic exposure but also the airborne route of allergen exposure of atopic patients. In these mice, allergic rhinitis could be demonstrated by analysis of different parameters of the inflammatory spectrum: IL-5 and eosinophils in NLF, histological evidence of eosinophilic inflammation and *in vivo* increased mucosal thickness on MRI scans. Moreover, subsequent acute allergen exposure of these mice after an interval of 10 days led to a systemic reaction as evidenced by increased IL-5 levels in serum and eosinophils in blood, with concomitant re-induction of eosinophilic inflammation in nasal tissue and eosinophilic influx in both upper and lower airway lumen.

Two new techniques were developed to demonstrate nasal inflammation in mice. First, nasal lavage was developed to investigate cellular and cytokine contents in nasal secretions. Analyses of NLF showed infiltration of eosinophils in mice that inhaled OA, and the percentage of eosinophils (8.6%) in NLF of mice was comparable to that in patients with allergic rhinitis [16,17]. Moreover, eosinophil count in NLF is a valuable parameter as it correlated well with the histological eosinophil count in nasal mucosa. Dependent on the aims of studies, one could consider omitting histological analyses in future studies because histological examination is more time-consuming and laborious than nasal lavage. On the other hand, the murine nose is small and thus the area of inflammation limited, which results in low numbers of cells in NLF. Despite their dilution by the lavage fluid, cytokines could still be detected in NLF at 6 h after acute provocation. Apart from IL-5, also IL-4 levels (data not shown) were maximal at 6 h after provocation.

The technique of nasal lavage in mice therefore emerges as a valuable method for evaluating the content of nasal secretions and it represents a new parameter for research on allergic rhinitis. In addition to nasal lavages, MRI scans of the murine skull are a new, non-invasive but technically demanding tool for investigating the nasal mucosa *in vivo*. An increased thickness of the nasal mucosa was observed at the superior lateral nasal wall of OA-sensitized and challenged mice. This phenomenon was demonstrated here to be allergen-specific and was consistent with the inflammation found on histology. Apart from the mucosal swelling in OA mice, no other abnormalities or signs of

sinusitis, commonly observed in asthmatics [18] were found in these mice. More prolonged allergen exposure or contact with pathogenic microorganisms might be required for the development of sinusitis.

Interestingly, allergic inflammation was simultaneously and progressively induced in the upper and lower airways in the chronic model with daily allergen exposure for 8 days, and parameters of inflammation were positively correlated in the upper and lower respiratory tract. In spite of the predominant deposition of inhaled allergens in upper airways (83%, unpublished observation), the degree of inflammation was most pronounced in lower airway tissues. Similar observations were made in humans by Chanez *et al.* [5] who found a higher degree of eosinophilic inflammation in the lower airways of allergic asthmatic patients with rhinitis.

In order to mimic the exacerbation of respiratory symptoms by intermittent allergen exposure, we performed an acute allergen provocation of pre-sensitized and chronically exposed mice after an allergen-free interval of 10 days. A renewed induction of eosinophilic inflammation was found in nasal tissue, where the inflammatory response was maximal at 6 h after provocation and subsided from 12 h onwards. In both NLF and BALF, eosinophils reached their peak concentration at 6 h after provocation, which coincided with the peak level of IL-5 in NLF, BALF and serum. Surprisingly, no increased peribronchial inflammation was found for up to 72 h after provocation.

In view of the major deposition of inhaled allergen in the nose, the nose might be primarily involved in eliciting the systemic allergic response, resulting in lower airway inflammation. A systemic response, characterized by increased circulating levels of IL-5 and bone-marrow eosinophilia, has already been described after intranasal application of OA in sensitized mice [19]. Also in humans, a systemic allergic response has recently been suggested after segmental bronchial provocation, resulting in enhanced blood eosinophilia and nasal eosinophilic inflammation [20]. Taken together, these data point to a remarkable systemic response following allergen encounter. In spite of the systemic increase in IL-5 in our study, no renewed peribronchial tissue inflammation was found after a single provocation. This finding suggests that induction of lower airway inflammation requires a chronic exposure or a higher dose of allergen, while upper airways are more sensitive to a single exposure.

Our murine model of allergic asthma and rhinitis offers a particularly valuable investigative tool for studying pathophysiological mechanisms and for testing potential therapies for allergic airway diseases. As our results show for the first time simultaneous induction of eosinophilic inflammation in the murine nose and lung after inhalation of aerosolized allergen, they support the viewpoint that upper and lower

airway diseases represent a continuum of inflammation involving one common airway. The coexistence of rhinitis and asthma in patients with respiratory allergy has important implications for managing airway allergy.

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