

Eotaxin-2 and eotaxin-3 expression is associated with persistent eosinophilic bronchial inflammation in patients with asthma after allergen challenge

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Background: Eotaxin-1, eotaxin-2, and eotaxin-3 are chemokines involved in the activation and recruitment of eosinophils through activation of their main receptor, CC chemokine receptor 3. The differential roles of these chemokines still remain to be established. It has been suggested that eotaxin-1 is an important mediator in the early phase of allergen-induced recruitment of eosinophils into the airways. Eotaxin-2 and eotaxin-3 might play a role in the subsequent persistence of allergen-induced bronchial eosinophilia. **Objective:** The aim of this study was to determine the expression of eotaxins and eosinophil counts in the bronchial mucosa of subjects with mild asthma after resolution of the late-phase asthmatic response (LAR).

Methods: The expression of eotaxins and eosinophil counts were determined in bronchial biopsy specimens obtained from 10 subjects with mild asthma 48 hours after diluent and allergen challenge by using immunohistochemistry. Positively stained cells were counted in a 125- μ m-deep zone of the lamina propria. **Results:** Eotaxin-2 and eotaxin-3 expression in bronchial mucosa was significantly increased 48 hours after allergen challenge ($P = .001$ and $P = .013$, respectively). At this time point, when marked tissue eosinophilia was still present, these increases were positively correlated with the magnitude of the LAR ($r = 0.72$, $P = .019$ and $r = 0.64$, $P = .046$, respectively). Furthermore,

eotaxin-2 expression was associated with the number of eosinophils after allergen challenge ($r = 0.72$, $P = .018$).

Conclusion: Our findings suggest that eotaxin-2 and eotaxin-3 might account for the persistence of bronchial eosinophilia after resolution of the LAR. (J Allergy Clin Immunol 2005;115:779-85.)

Key words: Asthma, allergen, eotaxin-1 (CCL11), eotaxin-2 (CCL24), eotaxin-3 (CCL26), eosinophil, chemokines, bronchial biopsy, airways inflammation

Asthma is an inflammatory disease of the airways characterized by an increased number of eosinophils in the bronchial mucosa. Most allergic and nonallergic asthmatic subjects, including subjects with mild asthma, have bronchial eosinophilia, which is associated with asthma severity and airway hyperresponsiveness.^{1,2} Even in patients in clinical remission of the disease, increased numbers of eosinophils and other inflammatory cells have been observed in the airways.³ The mechanisms and mediators involved in the persistence of airway eosinophilia in asthma are not fully understood. Bronchial exposure to allergen can induce a late-phase reaction that is accompanied by local recruitment and activation of eosinophils in bronchial biopsy specimens.⁴ The late-phase reaction has been considered as a model system to study the mechanisms of asthmatic airways inflammation.

Numerous chemoattractants, including lipid mediators (platelet-activating factor and leukotrienes), cytokines, and various chemokines, can mediate the migration of eosinophils into tissues. Eotaxins are members of the CC chemokine family that are potent attractants of eosinophils and thereby might contribute to allergic inflammation. To date, 3 eotaxins have been identified: eotaxin-1 (CCL11), which is located on chromosome 17q11 in the CC chemokine cluster,^{5,6} and eotaxin-2 (CCL24)^{7,8} and eotaxin-3 (CCL26),^{9,10} both of which were mapped to chromosome 7q11. They have comparable functional properties and mainly bind to CC chemokine receptor 3 (CCR3), which is expressed by several inflammatory cell types, including eosinophils, mast cells, and macrophages.¹¹ Nevertheless, the sequence similarity of the eotaxins is less than 40%, and they differ almost entirely in the amino-terminal region.

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Abbreviations used

CCR3: CC chemokine receptor 3
 EAR: Early-phase asthmatic response
 LAR: Late-phase asthmatic response

The functional effects of eotaxin-1 in eosinophils not only include chemotaxis of eosinophils to the site of inflammation¹² but also mobilization of eosinophils and their progenitors from the bone marrow¹³ and enhancement of adhesion of eosinophils to the endothelium.^{6,7,14-16} Finally, eotaxin-1 can cause these cells to degranulate and thus potentially contributes to tissue damage.¹⁷ The interaction between eotaxin-1 and CCR3 is one mechanism that might explain the selective recruitment of eosinophils to sites of allergic inflammation.

Several studies have implicated eotaxin-1 and CCR3 in the pathogenesis of asthma. It has been shown that mRNA and protein expression of both eotaxin-1 and CCR3 in bronchial biopsy specimens was significantly higher in asthmatic subjects than in healthy volunteers.¹¹ After allergen challenge, eotaxin-1 expression correlated with the early phase of eosinophil recruitment into the airways during the late asthmatic response.¹⁸ Eotaxin-1 is also associated with early tissue eosinophilia in the skin after allergen challenge.¹⁹ However, in both studies this chemokine does not appear to account for the persistence of tissue eosinophilia after allergen exposure because its expression was decreased at later time points.

Less is known about the biologic roles and kinetics of eotaxin-2 and eotaxin-3 in the asthmatic allergic reaction. More recently, it has been suggested that these 2 chemokines could be involved in the persistence of allergen-induced eosinophilic inflammation. This is based on the observations that eotaxin-2 mRNA expression was significantly increased 24 hours after allergen exposure of the skin in atopic subjects¹⁹ and that eotaxin-3 mRNA expression was markedly increased in the airways of asthmatic patients 24 hours after allergen challenge.²⁰

We hypothesized that eotaxin-2 and eotaxin-3 are important for the ongoing tissue eosinophilia that is present after resolution of the late-phase asthmatic response (LAR). Our primary objective was to measure the protein expression of the 3 eotaxins in conjunction with eosinophil counts in bronchial biopsy specimens obtained 48 hours after diluent and allergen challenge in patients with mild asthma. As a secondary objective, we determined whether expression of the eotaxins was associated with the presence of mast cells or macrophages.

METHODS

Subjects

The current study was part of a larger project, part of which has been published previously.²¹ Ten nonsmoking subjects with mild intermittent asthma²² requiring only the intermittent use of β -agonist therapy participated. All individuals were clinically stable, were sensitive to house dust mite, had baseline FEV₁ values of greater than

70% of predicted value,²³ were hyperresponsive to inhaled histamine (PC₂₀ <8.0 mg/mL),²⁴ and demonstrated both an early-phase asthmatic response (EAR) and an LAR (EAR: decrease in FEV₁ of $\geq 20\%$ from baseline 0-3 hours after allergen; LAR: decrease in FEV₁ of $\geq 15\%$ from baseline 3-7 hours after allergen) to inhaled house dust mite extract in the screening period.^{21,24} The Medical Ethics Committee of the Leiden University Medical Center granted approval for the study, and all patients provided prior written informed consent.

Study design

The study had a randomized, placebo-controlled, crossover design. Bronchoscopy was performed in each individual 48 hours after allergen and diluent challenge. Challenges were separated by a washout period of at least 2 weeks.

Allergen challenge and bronchoscopy

Allergen challenge was performed according to a standardized protocol by using purified aqueous allergen extract of *Derma-tophagoides pteronyssinus* (SQ 503; Vivodiagnost, ALK Abello, Nieuwegein, The Netherlands).²⁴ Consecutive doubling concentrations of allergen were inhaled during tidal breathing for 2 minutes with the nose clipped at 10-minute intervals until a decrease in FEV₁ of 20% or more from baseline value (after diluent) was reached. The response to allergen was measured in duplicate 10 minutes after each inhalation of allergen and repeated 10, 20, 30, 40, 50, 60, 90, and 120 minutes and then hourly until 7 hours after the last inhalation. The magnitude of the EAR and LAR were calculated as the area under the curve (percentage decrease in FEV₁ multiplied by hour).

Fiberoptic bronchoscopy was performed according to a standardized and validated protocol.²⁵ Six biopsy specimens were taken at the (sub) segmental level, of which 3 were fixed in formalin for 24 hours and embedded in paraffin as previously described.²¹ Biopsy samples from this study by Ricciardolo et al²¹ were also used in the present study.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections. Hematoxylin and eosin staining was carried out to select 2 of 3 biopsy samples for each subject per challenge. We selected biopsy specimens that had a subepithelial area, excluding crush artifact, cartilage, blood clot, or fragments of glands.²⁶ All antibodies were diluted in 1% (wt/vol) BSA (Sigma-Aldrich Chemie, Munich, Germany)-PBS. Antigen retrieval was performed on paraffin-embedded sections with citrate (eotaxin-1, mast cell tryptase, and CD68), pepsin (eotaxin-2 and eotaxin-3), or trypsin (EG-2).²⁷ Sections were incubated at room temperature with mouse mAbs directed against eosinophils (1:600, clone EG-2; Pharmacia, Uppsala, Sweden), mast cell tryptase (1:16000 for clone AA1; DAKO, Glostrup, Denmark), CD68-positive macrophages (1:12000 for clone KP-1; DAKO), eotaxin-1, eotaxin-2, and eotaxin-3 (1:50 for clone 43911, 1:400 for clone 61016, and 1:75 for clone 115002, respectively; R&D Systems, Abingdon, United Kingdom). As a secondary antibody, the anti-mouse Envision System (DAKO) was used. Positive cells stained red after development with NovaRed (Vector Laboratories, Burlingame, Vt). Sections were counterstained with Mayer's Hematoxylin. Omission and substitution of the primary antibody with an isotype-matched control antibody of the same species was used as a negative control.

Assessment and quantification of immunohistochemical staining

For each subject, 2 of 3 biopsy specimens were selected per challenge, as described in the previous section. One observer was

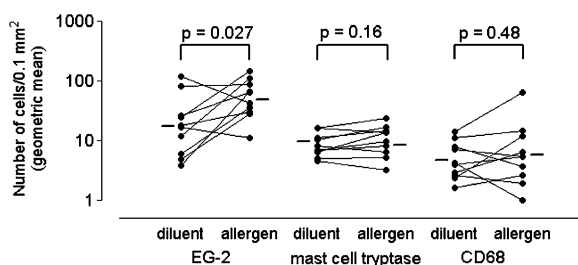


FIG 1. Individual numbers of cells positive for EG-2, mast cell tryptase, and CD68 in the submucosa of bronchial biopsy samples obtained 48 hours after diluent or allergen challenge in subjects with mild asthma. The data are expressed as the number of cells per 0.1 mm², and the geometric mean is depicted as a horizontal bar.

blinded to the patients' clinical status and examined all coded biopsy specimens under supervision of a pathologist. By using digital image analysis (KS400; Carl Zeiss B.V., Sliedrecht, The Netherlands),²⁸ all suitable submucosal area in each tissue section was selected as a 125-μm-deep zone beneath the basement membrane. During image acquisition of this zone, the areas of airway smooth muscle and gland tissue were excluded by drawing frames around the area to be quantified. Next, cells that stained positively for EG-2, mast cell tryptase, CD68, eotaxin-1, eotaxin-2, and eotaxin-3 were counted interactively in the framed selection of the submucosal area. Results were expressed as the total number of positive cells per 0.1 mm². The mean submucosal area per section that was analyzed was 0.29 mm² (range, 0.11-0.62 mm²).

Statistical analysis

The numbers of cells per 0.1 mm² were log transformed before analysis and expressed as the geometric mean and range. Because the eotaxin-2 data contained values of 0, we added 1 to each value from this data set before log transformation. PC₂₀ histamine is also expressed as the geometric mean and range. All other data are reported as means ± SD. Statistical analysis was conducted with 2-tailed paired Student *t* tests to compare the 2 conditions (diluent and allergen). The Pearson coefficient was calculated to determine correlations.

RESULTS

Patient characteristics

The patients participated in a larger project, part of which has been published previously.²¹ Ten subjects with mild intermittent asthma,²² 5 male and 5 female subjects, were included (age, 22.1 ± 2.6 years). All subjects had normal lung function (baseline FEV₁, 94.4% ± 7.2% predicted) and were hyperresponsive to histamine (PC₂₀, 1.17 mg/mL [0.29-4.23 mg/mL]). There was no difference in baseline FEV₁ between the diluent and allergen challenge. All subjects exhibited an EAR and an LAR with maximum decreases in FEV₁ from baseline of 43.7% ± 11.3% and 33.2% ± 14.2%, respectively. Diluent challenge did not affect baseline FEV₁ at any given time point.

Eosinophils, mast cells, and CD68-positive macrophages

The numbers of EG-2-positive cells in the bronchial mucosa from the asthmatic patients were significantly increased 48 hours after allergen challenge compared with

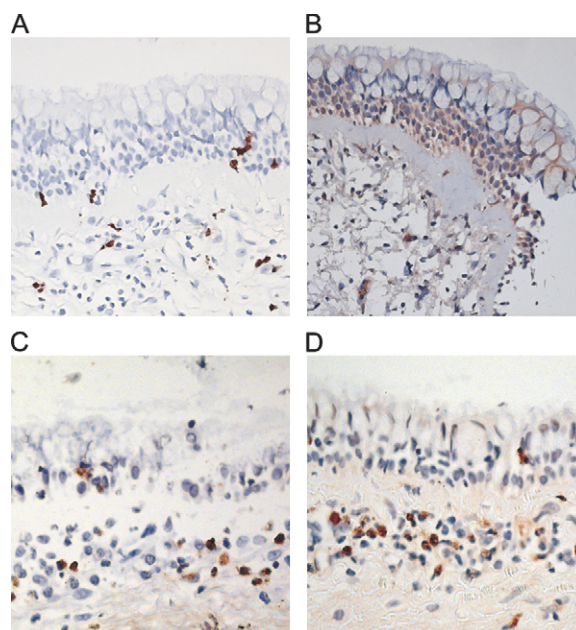


FIG 2. Immunohistochemical detection of EG-2 (A), eotaxin-1 (B), eotaxin-2 (C), and eotaxin-3 (D) in the submucosa of bronchial biopsy specimens of subjects with mild asthma 48 hours after allergen challenge (original magnification 200×). Positive cells are stained red.

those after diluent challenge ($P = .027$, Fig 1). However, the numbers of mast cells and CD68-positive macrophages did not differ significantly between the 2 challenges ($P = .16$ and $P = .48$, respectively; Fig 1).

Eotaxin-1, eotaxin-2, and eotaxin-3

Expression of eotaxin-1-, eotaxin-2-, and eotaxin-3-positive cells was detected as a granular staining pattern in the cytoplasm of inflammatory cells that infiltrated the submucosa and the epithelial layer (Fig 2). Epithelial cells stained clearly positive for eotaxin-1 and weakly positive for eotaxin-2 and eotaxin-3. In some biopsy samples there was weak staining of eotaxin-1 and eotaxin-3 in airway smooth muscle cells. Finally, eotaxin-3 was expressed by a fraction of the endothelial cells. A difference in expression pattern in epithelial or endothelial staining after diluent or allergen challenge was not detected.

Bronchial biopsy specimens from asthmatic patients obtained after allergen challenge showed increased numbers of eotaxin-2- and eotaxin-3-positive cells in the submucosa compared with numbers after diluent challenge ($P = .001$ and $P = .013$, respectively). However, the increase in eotaxin-1-positive cells after allergen challenge did not reach significance ($P = .065$, Fig 3).

Relationship between eosinophils, mast cells, or CD68-positive macrophages and expression of eotaxins

The difference in eosinophil numbers between the 2 challenges (allergen minus diluent) did not correlate with the difference in cells that stained positive for eotaxin-1,

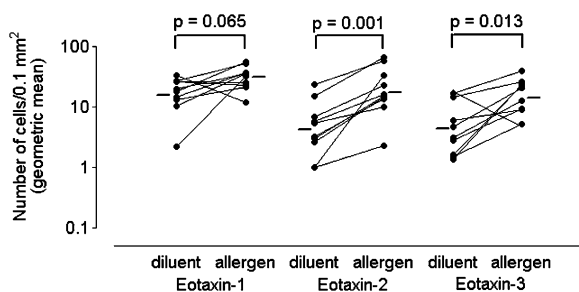


FIG 3. Individual values of eotaxin-1, eotaxin-2, and eotaxin-3 protein-positive cells in the submucosa of bronchial biopsy samples obtained from subjects with mild asthma 48 hours after diluent or allergen challenge. The data are expressed as the number of cells per 0.1 mm², and the geometric mean is depicted as a horizontal bar.

eotaxin-2, or eotaxin-3 (allergen minus diluent). The numbers of eosinophils detected after allergen challenge correlated positively with the numbers of cells expressing eotaxin-2 after allergen challenge ($r = 0.72$, $P = .018$) but not with eotaxin-1- or eotaxin-3-positive cells ($P > .05$, Fig 4). Neither mast cell numbers nor CD68-positive macrophage numbers correlated significantly with the numbers of eotaxin-1-, eotaxin-2-, or eotaxin-3-positive cells after allergen challenge (all $P > .05$).

Relationship between cells expressing eotaxins and the LAR

Interestingly, there was a significant positive correlation of the number of cells expressing eotaxin-2 or eotaxin-3 48 hours after allergen exposure with the magnitude of the LAR ($r = 0.72$, $P = .019$ and $r = 0.64$, $P = .046$, respectively; Figs 5 and 6). Such a correlation could not be found for eotaxin-1 ($P = .24$).

DISCUSSION

The results from the present study show that allergen exposure resulted in a significant increase of eotaxin-2 and eotaxin-3 expression in bronchial mucosa 48 hours after challenge in asthmatic patients. At this time point, when marked tissue eosinophilia was still present but mast cell or macrophage numbers were not affected, these increases were positively correlated with the magnitude of the LAR. Moreover, after allergen challenge, eotaxin-2 expression was associated with the number of eosinophils. Our findings suggest that eotaxin-2 and eotaxin-3 might account for the persistence of bronchial eosinophilia after resolution of the LAR.

To our knowledge, this is the first study in which protein expression of the 3 eotaxins in bronchial mucosa has been investigated 48 hours after allergen challenge in asthmatic subjects. We found that eotaxin-1 expression was slightly but not significantly increased at this time point. This might be due to the fact that allergen-induced eotaxin-1 expression had returned to near-baseline values at 48 hours after allergen challenge. This is in line with

several studies that demonstrated that eotaxin-1 expression peaked 4 to 6 hours after allergen challenge in the skin or airways and returned to baseline values at 24 hours.^{18-20,29} Moreover, eotaxin-1 protein levels in the airways of atopic asthmatic subjects during long-lasting clinical remission of the disease did not differ from levels found in the airways of healthy control subjects, despite ongoing eosinophilia.³ This suggests that eotaxin-1 is not critically involved in the persistence of eosinophilia that is present after resolution of the LAR or during clinical remission and that other chemokines or survival factors might be responsible for these processes.

We also observed a strong increase in eotaxin-2-positive cells 48 hours after allergen exposure. This is in line with the finding that eotaxin-2 mRNA expression peaked at 24 hours during a cutaneous late-phase response to allergen.¹⁹ However, another study reported that the number of eotaxin-2 mRNA-positive cells in bronchial biopsy specimens obtained from asthmatic patients was not changed 24 hours after allergen challenge.²⁰ Kinetic studies with nasal polyps that were obtained from atopic and nonatopic patients showed that when stimulated *ex vivo* with IL-4, eotaxin-2 is released within 24 hours from stimulation, which sustained for 96 hours.³⁰ The results from this study suggest that there might be a long-lasting production of eotaxin-2 when IL-4 is released *in vivo*. In addition, it was shown recently that production of eotaxin-2 and eotaxin-3 by bronchial epithelial cells *in vitro* was induced by IL-4 or IL-13 alone, which might suggest that these 2 eotaxins could be secreted at sites of T_H2-dominant allergic inflammation. Interestingly, the production of eotaxin-2 and eotaxin-3 required continuous exposure to IL-4.³¹ The present study also demonstrates that eotaxin-3 protein expression is increased after allergen challenge. This is a further extension of the observation that eotaxin-3 mRNA levels were increased 24 hours after allergen challenge in asthmatic subjects.²⁰ Recently, it was shown that serum levels of eotaxin-3 are increased in patients with atopic dermatitis compared with levels seen in healthy control subjects. Moreover, these levels were associated with the clinical severity of the disease and correlated with eosinophil numbers in peripheral blood,³² which might indicate a role for eotaxin-3 in the persistence of eosinophilia during allergic disease. Finally, our findings show that the expression of both eotaxin-2 and eotaxin-3 was associated with the magnitude of the LAR and that the number of eotaxin-2-positive cells correlated significantly with tissue eosinophilia after allergen exposure. These results provide evidence for the involvement of these 2 chemokines during the later stages of allergen-induced inflammation and indicate that the 3 eotaxins might have distinct roles concerning the dynamics of eosinophil recruitment.

In the present study all subjects obtained a significant airway response to allergen challenge in terms of decrease of FEV₁ during both the EAR and the LAR. The crossover design used allowed careful comparison of the outcome parameters determined after allergen and diluent challenge. To study the eosinophilic inflammation in the

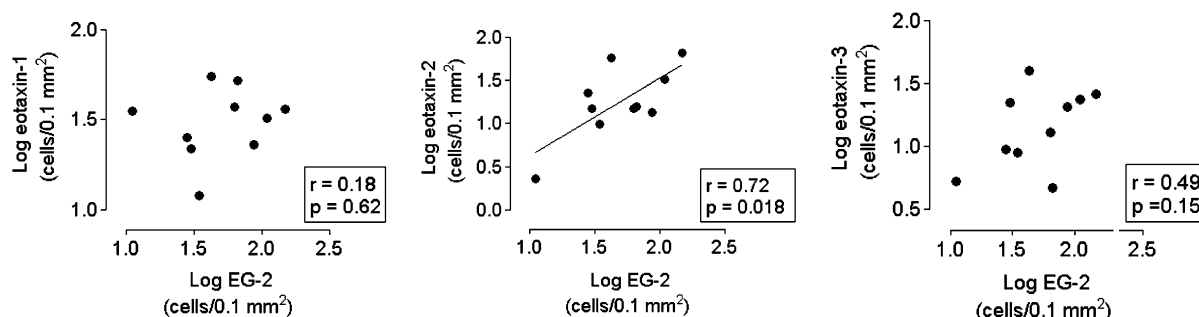


FIG 4. Relationship between the numbers of eotaxin-1-, eotaxin-2-, or eotaxin-3-positive cells and eosinophils in bronchial submucosa 48 hours after allergen challenge in subjects with mild asthma. The data are expressed as the logarithm of the number of cells per 0.1 mm².

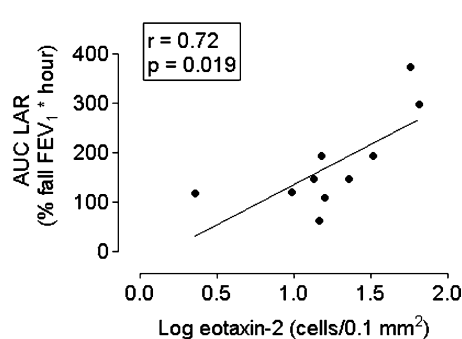


FIG 5. Relationship between the magnitude of the LAR and the number of eotaxin-2-positive cells in the bronchial submucosa 48 hours after allergen challenge in subjects with mild asthma. The LAR is measured as the area under the curve (AUC) and expressed as the percentage decrease in FEV₁ * hour. The number of eotaxin-2-positive cells is expressed as the logarithm of the number of cells per 0.1 mm².

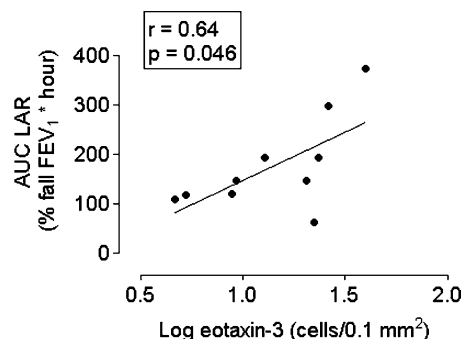


FIG 6. Relationship between the magnitude of the LAR and the number of eotaxin-3-positive cells in the bronchial submucosa 48 hours after allergen challenge in subjects with mild asthma. The LAR is measured as the area under the curve (AUC) and expressed as the percentage decrease in FEV₁ * hour. The number of eotaxin-3-positive cells is expressed as the logarithm of the number of cells per 0.1 mm².

bronchial submucosa that persists after resolution of the LAR, as observed in 7 of 10 subjects, the expression of eotaxins was measured 48 hours after allergen challenge. Whereas expression of all eotaxins was readily detectable in inflammatory cells, in epithelial cells we found a marked staining for eotaxin-1 but weak and variable staining for eotaxin-2 and eotaxin-3. *In vitro* studies have demonstrated that lung epithelial cells are a major source of eotaxin-1, eotaxin-2, and eotaxin-3 production.³³⁻³⁵ If epithelial cells do not store eotaxins intracellularly but release these chemokines immediately in the lung tissue, this could in part explain our results. A different possibility might be that eotaxin-2 and eotaxin-3 levels in the bronchial epithelium were below the detection levels of the immunohistochemistry procedures used.

The association between eotaxin-2 expression and the number of eosinophils 48 hours after allergen challenge might not necessarily indicate that the presence of eotaxin-2 expression explains the accumulation of eosinophils. However, this result supports the hypothesis that this chemokine could contribute to the sustained eosinophilic inflammation that was observed in the biopsy specimens. Because such a correlation was not found for eotaxin-3, it

could be argued that this chemokine is not important for the ongoing tissue eosinophilia that is present after resolution of the LAR. Another explanation is that although increased levels were found, eotaxin-3 is not the main regulator of eosinophil trafficking in the complex process of allergic inflammation in asthma that is obviously not dependent on just a single predominant mediator. Finally, it must be taken into account that using immunohistochemistry, we measured the sources of the production of eotaxins and not the gradients that are present within the lung tissue and regulate chemotaxis.

The signals that modulate the expression of eotaxins have been an active area of research. It has been demonstrated that the production of eotaxins can be induced by a range of proinflammatory cytokines in a variety of cell types, including resident cells in the airways, such as epithelial cells,^{31,33-35} fibroblasts,³⁶ endothelial cells,^{9,31} and smooth muscle cells,³⁷ and also infiltrating inflammatory cells, such as eosinophils³⁸ and monocytes.^{31,39,40} Various stimuli could result in differential regulation of production, availability, and activity of eotaxins among the cell types involved, whereas spatial and temporal regulation allow for the diversity of the allergic response.

This is illustrated by our recent observation that TGF- β 1 differentially modulates eotaxin-1 and eotaxin-3 expression induced by T_H2 cytokines in cultured airway smooth muscle cells.⁴¹ Whereas different stimuli have distinct effects on the expression of the various eotaxins, it has been shown *in vitro* that the eotaxins demonstrate activation of human eosinophils with similar efficacies for chemotaxis (although controversial results have been reported^{9,10,36}), cytoskeletal rearrangements, activation of Gi proteins and transients of [Ca²⁺]_i but a distinct profile of activity with respect to the binding to CCR3 and the release of reactive oxygen species.³⁶ Functional consequences of differentially regulated activation and expression of eotaxins *in vivo* are yet unknown. Determining the significance of the 3 eotaxins in the pathogenesis of allergic diseases has important implications for the design of therapeutic interventions aimed at blocking their activity. To date, animal studies have shown that targeting eotaxin-1 alone with specific neutralizing antibodies or by means of deletion of its gene does not result in a relevant reduction of allergen-induced airway eosinophilia or bronchial hyperresponsiveness.⁴²⁻⁴⁴ The specific consequences of blocking eotaxin-2 and eotaxin-3 *in vivo* have not yet been investigated. When a single chemokine does not provide a critical signal for recruitment and activation of inflammatory cells, therapy should be aimed at targeting the relevant chemokine receptor or receptors. Blockade of CCR3 with a specific neutralizing antibody or an antagonist impaired recruitment of eosinophils to the lung and to the skin.^{45,46} A previous animal study reported that the use of an antibody directed against CCR3 resulted in the depletion of eosinophils from the lung, such that the airway lumen was essentially devoid of eosinophils, and perivascular-peribronchial eosinophil numbers were reduced. This was accompanied by a significant decrease in mucus accumulation and airway hyperresponsiveness.⁴⁷ Taking into account the possible differential roles of eotaxin-1, eotaxin-2, and eotaxin-3 in eosinophil recruitment, CCR3 seems to be a promising target for therapeutic intervention in asthma.

From the results of this study, we conclude that both eotaxin-2 and eotaxin-3 might be mediators of the chronicity of allergen-induced eosinophilic inflammation that still exists after resolution of the LAR in subjects with mild asthma.

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