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Interactions between eotaxin and interleukin-5 in the chemotaxis of primed and non-primed human eosinophils

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Abstract

This study was designed to understand the relationship between interleukin-5 and eotaxin in modulating the chemotaxis of eosinophils obtained from healthy subjects and subjects with allergic rhinitis. Chemotaxis of eosinophils from patients with allergic rhinitis toward interleukin-5 (0.25 ng/ml) was 78% higher than that of healthy subjects. Incubation of eosinophils with eotaxin (100 ng/ml) did not change the interleukin-5-induced chemotaxis of eosinophils from healthy subjects, but it reversed the enhanced chemotaxis seen in eosinophils from allergic patients. Chemotaxis of eosinophils from patients with allergic rhinitis toward eotaxin (100 ng/ml) was 65% higher than that of eosinophils from healthy subjects. Incubation of eosinophils with interleukin-5 (100 ng/ml) significantly increased the eotaxin-induced chemotaxis in both subject groups, but such increases were markedly higher for cells from patients with allergic rhinitis. Our finding that eotaxin inhibits the enhanced eosinophil chemotaxis toward interleukin-5 in primed cells suggests that this chemokine may downregulate eosinophil accumulation in the nasal mucosa of allergic patients.

Keywords: Allergic rhinitis; Eosinophil chemotaxis; Interleukin-5; Eotaxin; Fibronectin

1. Introduction

Allergic diseases of the airways, such as asthma and allergic rhinitis, are inflammatory disorders that differ mainly in the sites of the inflammatory reaction and clinical manifestations. Allergic rhinitis is an inflammatory disease of the upper airways that is characterized by the accumulation of eosinophils in the nasal mucosa of patients (Quraishi et al., 2004). Eosinophils interact in a sequential order with endothelium and interstitial extracellular matrix components, such as fibronectin, resulting in their extravascular migration and accumulation at inflammatory sites (Kuna et al., 1998; Hanazawa et al., 2000). The early steps in the movement of eosinophils from the peripheral blood to tissues take place via the binding of the integrin family of adhesion molecules, the β_1 subfamily of very late antigen (VLA) 4 ($\alpha_4\beta_1$; CD49d/CD29) as well as the Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) member of the β2 subfamily, with the vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1), respectively (Wardlaw, 2001).

A number of chemokines have been shown to attract eosinophils with variable degrees of selectivity, including the subfamily of CC-chemokines, amongst which eotaxin (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26) play important roles (Shahabuddin et al., 2000; Teran, 2000; Kaplan, 2001). Increased expression of eotaxin has been demonstrated in the airways of subjects with asthma and in the nasal mucosa of subjects with allergic rhinitis, suggesting an important role in allergic diseases (Lamkhioued et al., 1997; Minshall et al., 1997). In addition, increased levels of eotaxin have been detected in the serum/plasma (Jahnz-Ro et al., 2000; Tateno et al., 2004) and sputum (Yamamoto et al., 2003) of asthma patients. Eotaxin contributes to selective eosinophil chemotaxis and transendothelial migration via the eotaxin receptor CCR-3, responses which are primarily mediated by β_2-integrins (Tachimoto et al., 2002). For instance, in eosinophils prestimulated with interleukin-5, eotaxin upregulates the CD11b/CD18

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molecule, leading to increased adhesion to fibronectin in vitro (Lundahl et al., 1998).

In healthy individuals, eosinophils adhere to activated endothelial cells, but do not transmigrate through this layer. Prior treatment with a cytokine such as interleukin-5 enables the eosinophils to migrate through the endothelium. In contrast, in allergic individuals, eosinophils not only adhere to but pass spontaneously through the endothelial cell layer, suggesting that priming of circulating eosinophils is a fundamental prerequisite for endothelium transmigration (Lampinen et al., 2004). Moreover, in guinea pigs and mice, eotaxin and interleukin-5 act co-ooperatively to promote the recruitment of eosinophils into tissues (Mould et al., 1997; Palframan et al., 1998; Foster et al., 2001). It has been suggested that interleukin-5 participates in eosinophil mobilization from bone marrow, whereas eotaxin induces their trafficking to tissues (Palframan et al., 1998). The present study was designed to further understand the relationship between interleukin-5 and eotaxin in modulating eosinophil chemotaxis in vitro, comparing this functional response in cells from healthy individuals (non-primed) and from subjects with allergic rhinitis (primed).

2. Materials and methods

2.1. Patients

All subjects gave their written consent for participation in the study. The protocols were approved by the local Ethics Committee and were conducted in accordance with the Declaration of Helsinki. Briefly, peripheral blood leukocytes were obtained from two groups of volunteers grouped primarily on the basis of their atopic status: (1) 33 normal healthy non-atopic subjects with peripheral blood eosinophilia ranging between 2% and 5% of total leukocytes; and (2) 54 patients with allergic rhinitis with a peripheral blood eosinophilia ranging between 5% and 19% of total leukocytes who had been pre-screened for the presence of allergen-induced rhinitis symptoms (sneezing, rhinorrhea, and nasal congestion). Atopy was defined on the basis of positive skin prick test reactions to a panel of common aeroallergens, including house dust mite, animal danders, and grass pollen. None of the patients were receiving antihistaminics, steroids, or nonsteroidal anti-inflammatory drugs at the time of the study. For the chemotaxis and adhesion assays, 30 normal healthy non-atopic subjects and 20 patients with allergic rhinitis were selected. Serum interleukin-5 levels were measured in all the subjects.

2.2. Eosinophil isolation

Eosinophils were isolated from peripheral blood as described by Hansel et al. (1991), with minor modifications. Briefly, 60 ml of heparinized (20 U/ml) blood from healthy or allergic subjects was diluted 1:1 with phosphate-buffered saline (PBS) and 35 ml of diluted blood was overlaid onto a 15 ml Percoll gradient (1.088 g/ml, pH 7.4, 340 mosmol/kg H_2O). Gradients were centrifuged at 700 *g* for 20 min, 4 °C (Jouan, Saint-Herblain, France) and the red cell pellet was collected. Red cells in the granulocyte pellet were lysed with lysis buffer (155 mM NH4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Washed granulocytes were incubated with anti-CD16 immunomagnetic microbeads and then passed over a steel-matrix column in a magnetic field (Miltenyi Biotec Inc., Aubum, USA); CD16-negative eosinophils were collected. The final suspension contained 97–99% eosinophils and contaminating cells were neutrophils. Cell viability (>93%) was assessed in the trypan blue dye exclusion test. Before use, eosinophils were resuspended in Eagle's minimum essential medium (MEM), pH 7.2.

2.3. Chemotaxis assays

Eosinophil migration was measured using a 96-multiwell ChemoTx 101–5 chamber. The wells in the microplate (e.g., bottom compartment) were filled with 29 μl of chemotactic agent (eotaxin or interleukin-5) diluted in MEM. A polycarbonate filter (5 μm pore size) was positioned on the loaded microplate and secured in place with corner pins. Next, eosinophils (25 μl; 4×10 cells/ml) treated with either interleukin-5 (0.2 ng/ml) or eotaxin (100 ng/ml) stet placed directly onto the filter sites. Untreated eosinophils (incubated with MEM) were used as controls. The chamber was then incubated for 2 h at 37 °C in a humid atmosphere with 5% CO_2. After incubation, the non-migrating cells on the origin side (i.e., top) of the filter were removed by gently wiping the filter with a tissue and the chamber was centrifuged at 200 *g* for 5 min at 20 °C. The filter was then removed and the number of cells that had migrated into the bottom compartment was determined by measuring residual eosinophil peroxidase (EPO). Each experiment was carried out in triplicate.

2.4. Cell adhesion assays

The adhesion assay was performed as previously described (Conran et al., 2001). In brief, 96-well plates were prepared by coating individual wells with 60 μl of fibronectin (20 μg/ml in PBS) overnight at 4 °C. Wells were then washed twice with PBS before blocking non-coated sites with 0.1% (w/v) bovine serum albumin (BSA) for 60 min at 37 °C. Wells were washed twice again with PBS and the plates were allowed to dry. Eosinophils were added in a volume of 50 μl of MEM/ovalbumin (7×10^4cells/ml) to the coated wells of a 96-well plate. Cells were allowed to adhere to wells for 15 min at 37 °C in a humid atmosphere with 5% CO_2. After incubation non-adhered cells were removed and the remaining cells were washed twice with PBS. Fifty microliters of MEM was added to each well and varying concentrations of the original cell suspension (in MEM) were added to empty wells to form a standard curve. Eosinophil adhesion was calculated by measuring the residual eosinophil peroxidase (EPO) activity of adherent cells. Eosinophils were pre-incubated with eotaxin (100 ng/ml) or interleukin-5 (0.25 ng/ml) for 15 min at 37 °C, in a humid atmosphere with 5% CO_2 before the cells were added to fibronectin-coated wells.

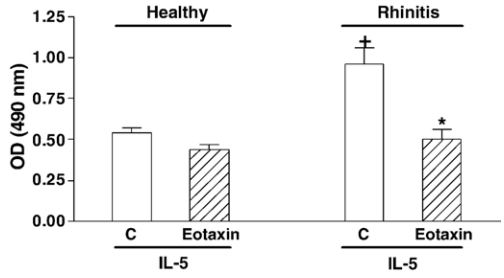

Fig. 1. Effect of eotaxin on chemotaxis toward interleukin-5 (IL-5; 0.25 ng/ml) of eosinophils obtained from healthy subjects and patients with allergic rhinitis. Eosinophils from both groups were incubated with or without eotaxin (100 ng/ml; right-ratched columns). The control value (C) represents the IL-5-induced chemotaxis in the absence of eotaxin (open columns). Each column represents the mean±S.E.M. from nine independent experiments for each group (three replicate in each). $^{+}P<0.05$ compared wth control (C) values for eosinophils from healthy subjects $*P<0.05$ compared with respective control (C) value.

2.5. Eosinophil peroxidase (EPO)

Fifty microliters of EPO substrate (1 mM H_2O_2, 1 mM *o*-phenylenediamine and 0.1% triton X-100 in Tris buffer pH 8.0) was added to each well. After a 30 min incubation at room temperature, 25 µl of 4 M H_2SO_4 was added to each well to stop the reaction and absorbance was measured at 490 nM in a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, USA). Adherence was calculated by comparing absorbance of unknown cells samples from to that of the standard curve (eosinophil number versus EPO activity) for cells from healthy subjects and rhinitis patients. The standard curves for cells from healthy subjects and rhinitis patients did not differ significantly, indicating that EPO activity can be used as a marker of eosinophil number.

2.6. Serum interleukin-5 levels

Interleukin-5 was measured in serum with commercially available enzyme-linked immunosorbent assay (ELISA) kits, following the instructions of the manufacturer (DuoSet, R&D, Minneapolis, USA). The lowest detectable concentration of interleukin-5 was 23.4 pg/ml.

2.7. Materials

The VarioMACS system (with columns and microbeads) and 96-multiwell ChemoTx 101–5 chamber were obtained from Miltenyi Biotec (Auburn, CA, USA) and Neuroprobe (Gaithersburg, USA), respectively. Eotaxin, interleukin-5, fibronectin and *o*-phenylenediamine were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Percoll was purchased from Pharmacia (Uppsala, Sweden).

2.8. Statistical analysis

Data are expressed as the means±S.E.M of *n* experiments and were analyzed Kruskal–Wallis nonparametric analysis of variance (ANOVA) followed by Dunn's multiple comparisons test, or the Mann–Whitney test. A *P* value of <0.05 was taken as significant.

3. Results

3.1. Interleukin-5 and eotaxin-induced chemotaxis and viability in eosinophils from healthy subjects

Concentration–response curves for interleukin-5 and eotaxin were recorded using eosinophils obtained from healthy subjects. Significant eosinophil chemotaxis was observed for both interleukin-5 (0.60±0.04, 0.56±0.02 and 0.53±0.04 for 0.25, 0.5 and 1 ng/ml, respectively; $n=4$) and eotaxin (0.34±0.03, 0.48±0.03 and 0.45±0.05 for 50, 100 and 500 ng/ml, respectively; $n=4$), compared with random migration (non-stimulated cells; 0.15±0.02). In addition, eosinophil viability (Trypan blue exclusion assay) after 120 min of incubation with either interleukin-5 or eotaxin was >95% for all concentrations used ($n=9$). In further studies, interleukin-5 and eotaxin were routinely used at concentrations of 0.25 and 100 ng/ml, respectively.

3.2. Effect of eotaxin on interleukin-5-induced eosinophil chemotaxis

Fig. 1 shows that the chemotactic response of eosinophils from patients with allergic rhinitis toward interleukin-5 was 78% higher ($P<0.05$) than that of eosinophils from healthy subjects ($n=9$ for each group). Incubation of eosinophils from healthy subjects with eotaxin did not promote any significant alteration in the interleukin-5-induced eosinophil chemotaxis. In contrast, eotaxin reversed the enhanced IL-5-induced chemotaxis of eosinophils from patients with allergic rhinitis, bringing the chemotaxis back to levels observed with eosinophils from healthy subject.

3.3. Effect of interleukin-5 on eotaxin-induced eosinophil chemotaxis

Chemotaxis toward eotaxin was 65% higher ($P<0.05$) in eosinophils from patients with allergic rhinitis than in eosinophils from healthy subjects (Fig. 2; $n=9$ for each

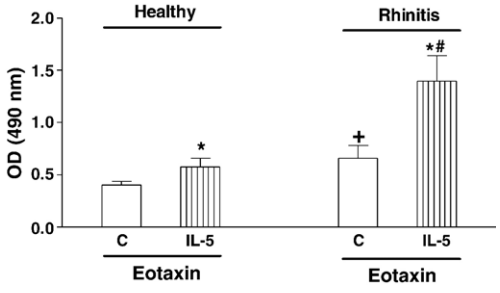

Fig. 2. Effect of interleukin-5 (IL-5) on chemotaxis towards eotaxin (100 ng/ml) of eosinophils obtained from healthy subjects and patients with allergic rhinitis. Eosinophils from both groups were incubated with or without IL-5 (0.25 ng/ml; striped columns). The control value (C) represents eotaxin-induced chemotaxis in the absence of IL-5 (open columns). Each column represents the mean±S.E.M. from nine independent experiments for each group (three replicates in each). $*P<0.05$ compared with the respective control (C) values; $^{+}P<0.05$ compared with control (C) values for eosinophils from healthy subjects; $^{\#}P<0.05$ compared with eosinophils from healthy subjects incubated with IL-5.

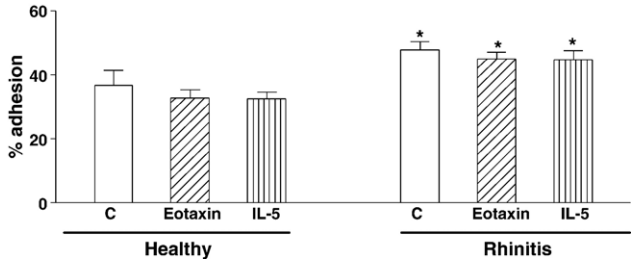

Fig. 3. Effect eotaxin and interleukin-5 (IL-5) on adhesion of eosinophils from healthy subjects and patients with allergic rhinitis to fibronectin-coated plates. Eosinophils were incubated with eotaxin (100 ng/ml; right-ratched columns) or IL-5 (0.25 ng/ml; striped columns) before cells were allowed to adhere to fibronectin-coated plates. The control value (C) is migration in the absence of IL-5 or eotaxin (open columns). Results are expressed as the mean percentage of adhered cells relative to the total number of cells±S.E.M. for nine different experiments group (three replicates in each). *$P<0.05$ compared with eosinophils from healthy subjects incubated with MEM (C), eotaxin or IL-5.

group). Incubation of eosinophils with interleukin-5 significantly increased the eotaxin-induced chemotaxis of eosinophils from both allergic and healthy individuals, but this increase was higher in cells from allergic patients.

3.4. Adhesion of eosinophils to fibronectin-coated plates: effect of interleukin-5 and eotaxin

Eosinophils from healthy subjects and patients with allergic rhinitis were pre-incubated with either eotaxin or interleukin-5 and allowed to adhere to fibronectin-coated plates. Fig. 3 shows that the basal eosinophil adhesion to fibronectin was 30% higher ($P<0.05$) in cells from allergic patients than in cells from healthy subjects ($n=9$ for each group). Prior incubation of eosinophils with either eotaxin or interleukin-5 did not significantly affect the adhesion of cells to fibronectin in either group.

3.5. Serum interleukin-5 levels

Interleukin-5 was detectable in the serum of 11 out of 54 patients with allergic rhinitis at a median concentration of 283.0± 107 pg/ml (ranging from 48 to 1151 pg/ml). Interleukin-5 was undetectable in the serum of all healthy subjects ($n=33$).

4. Discussion

Eosinophils from allergic patients exhibit increased chemotactic responses compared with those from normal subjects, which may reflect their primed state in such disorders (Warringa et al., 1992; Sehmi et al., 1992; Bruijnzeel et al., 1993; Ferreira et al., 2002). Priming is the state of activation resulting from exposure to a certain agent that itself has no effect on function but causes a more pronounced response to a subsequent, second agent (Lundahl et al., 1998). Since interleukin-5 levels are increased in the blood of allergic subjects (Walker et al., 1991; Till et al., 1995), it is suggested that this cytokine plays a key role in the enhancement of eosinophil chemotaxis. The mechanism of priming by interleukin-5 has been mimicked *in vitro* by using recombinant interleukin-5, resulting in enhanced adhesion to epithelial cells, chemotaxis, and degranulation (Carlson et al., 1992; Håkansson and Venge, 1994; Sanmugalingham et al., 2000). Furthermore, investigations with guinea pigs and mice suggest that interleukin-5 and eotaxin act co-operatively to selectively and synergistically promote eosinophil recruitment into tissues (Mould et al., 1997; Palframan et al., 1998). The synergism between these two cytokines seems to involve the ability of eotaxin to selectively recruit eosinophils into tissues and of interleukin-5 to prime eosinophils, thus amplifying the responses to eotaxin (Lamkhioued et al., 1997). Nevertheless, the mechanisms by which eotaxin interferes with eosinophil responses have not yet been elucidated, since the mere production of eotaxin is not associated with eosinophil accumulation (Yamamoto et al., 2003). Moreover, eosinophil recruitment occurs solely under conditions in which Th2 cytokines and eotaxin are co-produced (Li et al., 1998; Rothenberg, 1999). In the present study, we designed experiments to further our understanding of the interactions between interleukin-5 and eotaxin that modulate *in vitro* eosinophil chemotaxis and adhesion in non-primed (healthy subjects) and primed cells (rhinitis patients). Our results showed that the basal chemotactic responses of eosinophils to interleukin-5 and eotaxin were higher in cells from allergic patients than in cells from healthy subjects. Moreover, contact with interleukin-5 further increased the eotaxin-induced chemotaxis of eosinophils from both healthy individuals and patients with allergic rhinitis, but the increase was greater for eosinophils from the allergic patients. Therefore, it is reasonable to assume that the primed eosinophil phenotype observed in allergic subjects may be mimicked by prior treatment of eosinophils from healthy subjects with interleukin-5. Circulating interleukin-5 in patients with atopic dermatitis has been shown to prime eosinophils, thus enhancing their migration to a number of chemoattractants *in vitro* (Bruijnzeel et al., 1993). In contrast, Sehmi et al. (1992) found that prior incubation of eosinophils from subjects with eosinophilia (associated with asthma) with interleukin-5 failed to enhance the chemotactic responses to platelet-activating factor (PAF), leukotriene B_4 (LTB_4) or *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), suggesting that *in vivo* pre-exposure of eosinophils to interleukin-5 leads to a refractoriness of eosinophils to the migration. Moreover, Warringa et al. (1992) found that pre-exposure of eosinophils to interleukin-5 enhanced fMLP-induced chemotaxis but reduced GM-CSF-induced responses, suggesting that interleukin-5 modulates eosinophil chemotaxis by selective upregulation or downregulation of chemotatic responses depending on the chemotactic agent used. Thus, besides the diversity of chemoattractants, it appears most likely that differences in the methods used for cell purification (metrizamide, Percoll, or CD16-immunomagnetic beads), the types of allergy studied (asthma, rhinitis, or dermatitis), and current treatment of patients with β_2-agonists and/or inhaled corticosteroid account for the discrepancy in results described above. Nevertheless, the finding that interleukin-5 does not prime eosinophils to IL-8 responses in allergic patients suggests that interleukin-5 may act as an essential cofactor (but not as the only cytokine) responsible for the *in vivo* priming of eosinophils (Lampinen et al., 1999).

In our study, the enhanced interleukin-5-induced chemotaxis seen for the eosinophils from the patients with allergic rhinitis was

reversed by incubation with eotaxin, which brought chemotaxis back to the levels seen for eosinophils from healthy subjects. An increased serum level (Jahnz-Ro et al., 2000; Tateno et al., 2004) and expression of eotaxin in bronchoalveolar lavage and airways of individuals with asthma (Lamkhioued et al., 1997) as well as in the nasal mucosa of patients with allergic rhinitis (Minshall et al., 1997) has been reported, suggesting an important role for this CC-chemokine in allergic diseases. Eotaxin has been shown to induce transient internalization of the surface CCR-3 receptor on eosinophils, accompanied by partial degradation of CCR-3 (Zimmermann et al., 1999). This internalization is followed by the re-expression of CCR-3, which is partially dependent upon *de novo* protein synthesis, indicating that, in addition to stimulating eosinophils to undergo migration, eotaxin may also be involved in stopping eukocyte movement (Zimmermann et al., 1999). In this study the exposure of eosinophils to 100 ng/ml of eotaxin reduced the surface expression of CCR-3 by up to 76% at 3 h. Zimmermann and Rothenberg (2003) showed recently that eotaxin pretreatment inhibited subsequent CCR-3-mediated transepithelial migration, which is indicative of a functional desensitization. In our present study, using the eotaxin concentration (100 ng/ml) and incubation time (2 h) reported to induce CCR3 internalization (Zimmermann et al., 1999), we found that eotaxin exposure reduced the IL-5-induced migratory response of eosinophils from allergic patients, whereas the migratory response of cells from healthy individuals was only slightly reduced by eotaxin. This suggests that *in vivo* priming with IL-5 may modulate the phenomenon of CCR3 receptor internalization induced by eotaxin. It is plausible to speculate, therefore, that increased levels of eotaxin in allergic patients act to down-regulate the chemotactic responses to interleukin-5.

Data indicate that cytokines can regulate the expression and function of adhesion molecules. Eosinophil adhesion to fibronectin has been shown to be mediated by the β1 integrin CD49d/CD29 and the β2 integrin CD11b/CD18 (Anwar et al., 1994; Broide and Sriramarao, 2001; Conran et al., 2001). In our study we observed that the basal adhesion of eosinophils from allergic patients to fibronectin-coated plates was significantly higher than that fo eosinophils from healthy individuals, which may be due to an increased CD11b/CD18 expression on such cells as result of the *in vivo* priming by interleukin-5 (Lundahl et al., 1998; Chiba et al., 2005). Eotaxin has been shown to increase the adhesion of eosinophils to fibronectin by upregulating CD11b/CD18 on eosinophils from allergic patients 24 h after allergen inhalation provocation, but not before (Lundahl et al., 1998). More recently, Chiba et al. (2005) showed that eotaxin enhances CD18 expression in eosinophils from allergic patients, but not in eosinophils from healthy individuals, which may account for the difference in eosinophil responsiveness between the two groups. We found, in adhesion assays using fibronectin-coated plates, that adhesion of eosinophils from healthy individuals was not affected by in vitro incubation with eotaxin. In eosinophils from rhinitis patients, eotaxin and interleukin-5 did not further increase adhesion, suggesting that the *in vivo* priming was sufficient to produce a maximal increase of CD18 expression, masking any additional effect produced by *in vitro* exposure to these cytokines. It is well-established that eosinophils adhere to endothelium and interstitial extracellular matrix components before migrating toward sites of inflammation (Kuna et al., 1998; Hanazawa et al., 2000). Although the migratory response depends on prior cell adhesion, these phenomena do not necessarily run in parallel, and in fact increased adhesion in several experimental conditions may lead to an inhibition of migration (Kuijpers et al., 1993; Yamamoto et al., 1998; Conran et al., 2001; Ferreira et al., 2004; for review see Ferreira et al., 2006). This may explain our contrasting findings that eotaxin and interleukin-5 modified the eosinophil chemotaxis, but not adhesion.

With regard to serum interleukin-5 levels, our results showed that only 21% of the patients with allergic rhinitis had detectable levels of interleukin-5 and that levels were not detectable in the healthy subjects. This is consistent with previous studies reporting that interleukin-5 is not always measurable in all patients with asthma or allergic rhinitis, although there is agreement that interleukin-5 is not detectable in healthy volunteers (Corrigan et al., 1993; Alexander et al., 1994; Abdelnoor et al., 2002; Braunstahl et al., 2003). In addition to our limited knowledge regarding cytokine metabolism *in vivo*, several factors may explain the variability in serum interleukin-5 concentration in allergic patients, such as the inability of the assay to detect low concentrations, as well as the fact that serum interleukin-5 is increased mainly after allergen challenge and during disease exacerbation (Braunstahl et al., 2001). Nevertheless, a low concentration of interleukin-5 in the serum is apparently enough to prime eosinophils in allergic patients.

In conclusion, our results clearly show that eotaxin acts to reduce the chemotactic responses of interleukin-5-primed eosinophils, suggesting that this chemokine acts to downregulate eosinophil accumulation in the nasal mucosa of patients with allergic rhinitis.

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