

Identification of IL-5 and RANTES as the major eosinophil chemoattractants in the asthmatic lung

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The study was carried out to identify those molecules that are important in vivo in the attraction of eosinophil granulocytes to the lungs of patients with asthma. Asthmatic patients with birch pollen allergy had lavages performed before and during the pollen season, and the chemotactic activity of the bronchoalveolar lavage fluid was tested against normal eosinophils. The activity was significantly increased during the pollen season as compared with the activity before the pollen season ($p < 0.01$). Neutralizing antibodies to IL-2, IL-5 and IL-8, leukemia inhibitory factor, and to RANTES were added to the bronchoalveolar lavage fluid. Antibodies to IL-5 and RANTES, but not to IL-2 and IL-8 or leukemia inhibitory factor, significantly inhibited the chemotactic activity for eosinophils ($p < 0.001$). It is concluded that IL-5 and RANTES are important chemoattractants in the lungs of patients with allergic asthma. The effect of IL-5 may be that of a cofactor to the chemotactic molecules, of which RANTES may be one of the most important in allergic asthma. (J Allergy Clin Immunol 1996;97:1110-5.)

Key words: IL-5, RANTES, eosinophils, asthma, lungs

It has been known for a century that eosinophils are accumulated in the lungs of asthmatic subjects.¹ It is believed that the eosinophil plays a crucial role in the development of chronic bronchial asthma.² The mechanisms by which the eosinophils are attracted to the lungs are only partly understood and most likely involve several steps.^{3,4} One such step is the adhesion of eosinophils to lung endothelial cells by specific adhesion molecules, such as vascular cell adhesion molecule-1 on the endothelial cell and very late activation antigen-4 on the eosinophil. This step requires the upregulation of the adhesion molecules on both cells, which may be brought about by exposure to cytokines, such as IL-4 and IL-5. Another step is the production in the lungs of signals, which will stimulate the eosinophils to migrate through the endothelial barrier and out into the lung tissue.

Abbreviations used

BALF: Bronchoalveolar lavage fluid
LIF: Leukemia inhibitory factor

The presence of such chemoattractant signals has been known for quite some years, although the precise nature of these molecules has remained enigmatic.⁵ Indeed, several suggestions have been made on the basis of in vitro findings and include lipid molecules such as leukotriene B₄ and platelet activating factor; cytokines such as IL-2, IL-5, and IL-8; RANTES; and histamine, to mention some.⁶⁻¹¹ From chromatographic studies on serum and bronchoalveolar lavage fluid (BALF), it has become clear that there is considerable heterogeneity in molecular size of the chemotactic molecules and that the major activities are dependent on cofactors.^{5,12-15} Several clinical studies have clearly suggested the importance for the development of asthma of the production of such chemotactic activities, because the development of asthma does not seem to occur when the production of the chemotactic activities is inhibited.¹⁶⁻¹⁸

In this preliminary report we have collected BALF from subjects with allergic asthma during

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the birch pollen season and used antibodies to some of the candidate chemotactic molecules to test the possibility that one or more of the already known molecules might be involved. Indeed, we will demonstrate that two of the putative molecules, IL-5 and RANTES, seem to be of major importance.

METHODS

Patients

The patients were described in detail in a previous publication.¹⁷ In brief, eight patients allergic to birch pollen, four men and four women, with a mean age of 29 years (range, 12 to 37 years) participated in this study. Two were occasional smokers. The patients were followed up during the spring, and bronchoalveolar lavage was performed before and during birch pollen season.

Procedures

Measurement of eosinophil chemotactic activity was performed with 48-well microchamber chemotaxis chambers (Neuro Probe Inc., Cabin John, Md.) according to a modified Boyden chamber technique, as previously described.^{19,20} Granulocytes were isolated from heparinized blood from apparently healthy blood donors by dextran sedimentation,²¹ washed with physiologic saline solution, and diluted to a concentration of $1.5 \times 10^9/L$ in Gey's solution with human serum albumin (2 gm/L). Granulocytes in Gey's solution with human serum albumin were applied above the filter, and the samples of BALF, with or without antibody, diluted 1:2 were added below the filter. The filter pore size was 5 μm . The migration of eosinophils after 1 hour of incubation at 37°C was measured according to the leading-front method.²² Eosinophils were identified by staining with Chromotrope 2R (Sigma Chemical Co., St. Louis, Mo.).

Because we had limited volumes of BALF from individual patients, BALF samples obtained from the patients during the birch pollen season were pooled, and aliquots of this pool (i.e., 200 μl) were kept frozen at -70°C until use. The BALF was never used after having been refrozen.

The following anti-human antibodies were used: anti-IL-2, anti-IL-5, anti-IL-8, anti-leukemia inhibitory factor (LIF), and anti-RANTES. All antibodies were purchased from R&D Systems Europe Ltd. (Abingdon, U.K.). The stock solution for all antibodies was 1 mg/ml. Ten milliliters of antibody solution, at different dilutions as indicated, was added to 100 μl of BALF.

Statistics

Nonparametric statistics, paired and nonpaired, were used to evaluate statistical differences in chemotactic activity. All calculations were performed on a personal computer with Statistica software (Statsoft, Tulsa, Okla.).

Eosinophil chemotactic activity in BAL after addition of anti-IL-5

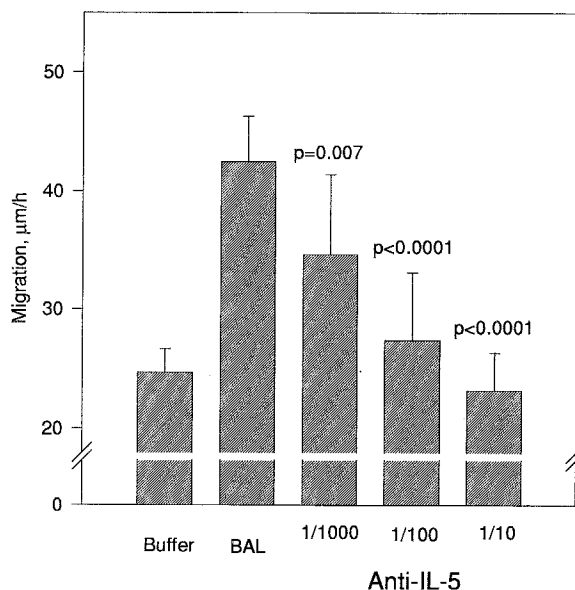


FIG. 1. Eosinophil chemotactic activity in BALF without and after the addition of various dilutions of anti-human IL-5. Migratory activity of the eosinophils toward buffer alone is shown for comparison. Effects of anti-IL-5 were evaluated by Wilcoxon's nonparametric test for paired samples. Data are expressed as means $\pm$ SD ($n = 12$). NS, Not significant.

RESULTS

The eosinophil chemotactic activity of the individual BALF samples was significantly increased during the birch pollen season as compared with activity before the pollen season ($p < 0.002$), as was shown before.¹⁷ The BALF-samples obtained from patients with asthma during the pollen season were pooled for the subsequent experiments, and the activity of the pool is shown in Figs. 1 to 3. The activity was significantly higher than with buffer alone ($p < 0.001$).

In Fig. 1 the consequences of the addition of antibodies to IL-5 to the BALF are shown. Anti-IL-5 progressively inhibited the eosinophil chemotactic activity of the BALF ($p < 0.001$). At a dilution of the IL-5 antibodies of 1:10, the chemotactic activity was completely inhibited and not different from that of buffer medium.

In Fig. 2 the consequences of the addition of antibodies to RANTES to the BALF are shown. Anti-RANTES progressively inhibited the eosinophil chemotactic activity of BALF ($p < 0.001$). However, the inhibition was never complete, and with a dilution of anti-RANTES of 1:10 the eosinophil chemotactic activity of BALF was still

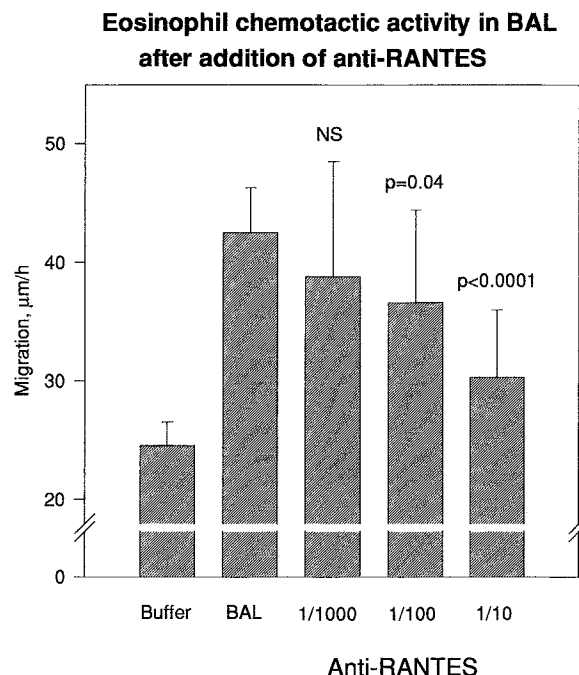


FIG. 2. Eosinophil chemotactic activity in BALF without and after the addition of various dilutions of anti-human RANTES. Migratory activity of the eosinophils toward buffer alone is shown for comparison. Effects of anti-RANTES were evaluated by Wilcoxon's nonparametric test for paired samples. Data are expressed as means $\pm$ SD ($n = 8$).

significantly increased as compared with buffer medium ($p = 0.005$).

In Fig. 3 the consequences of the addition of antibodies to IL-2 and IL-8 and to LIF to the BALF are shown. The eosinophil chemotactic activity of BALF was not changed with any of these antibodies.

To ensure that the antibodies by themselves did not have any negative effects on eosinophil migration, the antibodies were incubated together with the eosinophils, after which the migration of the eosinophils was measured. None of the antibodies affected random migration of the eosinophils (Table I), which most likely excludes any toxic effects of the antibodies. Nor did the antibodies exert any effects on eosinophil migration when added to the buffer medium below the filter (data not shown).

The specific inhibitory activity of anti-RANTES and anti-IL-5 was checked by the incubation of the respective antibody with RANTES and/or IL-5, after which the activity of the mixtures with respect to the attraction of eosinophils was tested ($n = 5$). The activity of the respective cytokine was completely inhibited by the corresponding antibody,

whereas no cross-inhibition of the activity of the other cytokine was noted (results not shown).

DISCUSSION

Earlier studies have shown a clear correlation in asthmatic subjects between the eosinophil chemotactic activity of BALF on the one hand and the number of eosinophils and asthmatic symptoms on the other.^{17, 23} These findings suggested the importance of the generation of chemotactic signals in the lungs for the development of asthma. Therefore in this study the finding of a complete abrogation of the chemotactic activity by antibodies to IL-5 and RANTES is of considerable interest and suggests that these two molecules are of major importance in the attraction of eosinophils to the allergic asthmatic lung. Recently, we have been able to measure, by means of a sensitive immunoassay, the concentration of RANTES in BALF from patients allergic to pollen. In most patients detectable levels were observed in the unconcentrated BALF, and rough estimates would indicate that RANTES is present in concentrations in the epithelial lining fluid of approximately 1 to 10 $\mu\text{g/L}$ (unpublished observations).

The mechanisms by which IL-5 and RANTES exert their effects cannot be conclusively determined by this study. However, the finding that antibodies to either of the molecules almost completely abrogated the chemotactic activity suggests that IL-5 and RANTES may operate by different mechanisms. It has recently been shown that recombinant IL-5 is a weak chemoattractant to eosinophils in vitro,²⁴ but rather acts as a chemokinetic principle.²⁵ This is in contrast to findings with RANTES, which showed RANTES to be a fairly potent chemotactic molecule to eosinophils in vitro.^{11, 26} The obvious interpretation of our findings in this study would therefore be that IL-5, like other chemokinetic molecules, acts as a necessary cofactor for the chemotactic molecules in BALF, whereas RANTES is a true chemotactic factor. It is interesting in this regard that it was impossible to inhibit all chemotactic activity in the BALF by anti-RANTES, which suggests that RANTES is not the only chemotactic molecule in the BALF of patients with allergic asthma.

The specificity of our results is demonstrated by the nonexistent effects of antibodies to some other cytokines. IL-8 has primarily been shown to be chemotactic to neutrophils²⁷⁻²⁹ but was also shown to be chemotactic to eosinophils in vitro.^{9, 30} The absence of any inhibition of the eosinophil chemotactic activity in BALF of patients with allergic

Eosinophil chemotactic activity in BAL after addition of various antibodies

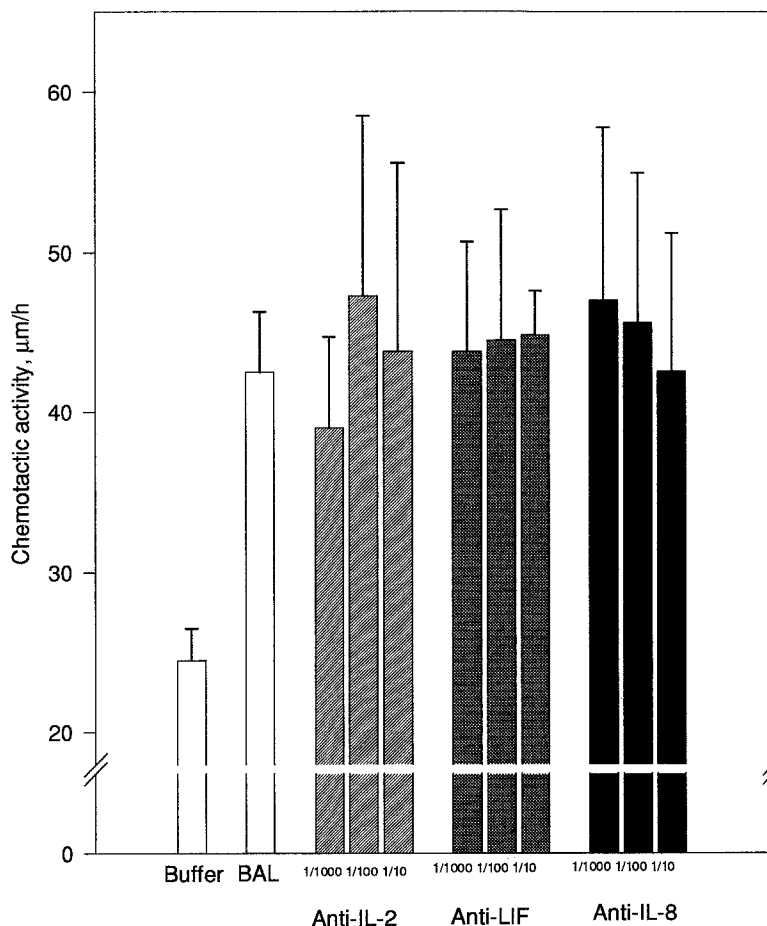


FIG. 3. Eosinophil chemotactic activity in BALF without and after the addition of various dilutions of anti-human IL-2, IL-8, and LIF. Migratory activity of the eosinophils toward buffer alone is shown for comparison. Effects of the antibodies were evaluated by Wilcoxon's nonparametric test for paired samples. Data are expressed as means $\pm$ SD ($n = 6$).

TABLE I. Effect of various antibodies on spontaneous migration of eosinophils

	Buffer	Anti-RANTES	Anti-IL-5	Anti-IL-2	Anti-IL-8	Anti-LIF
Mean	25.1	25.3	28.1	28.5	26.4	26.5
SD	1.0	3.9	2.1	2.6	1.5	3.0

Migration is expressed as micrometers per hour, and results given as means $\pm$ SD ($n = 6$). Wilcoxon's nonparametric test for paired samples was used. No significant differences were found.

asthma does not exclude the biologic importance of IL-8 as an eosinophil chemoattractant in other diseases. It should also be kept in mind that the target cells in our study were normal eosinophils. Thus the sensitivity of primed eosinophils, as found in patients with allergic asthma, may be quite different and is the subject of current studies. This may also be true for IL-2, which was shown to

be active at extremely low concentrations in vitro.¹¹ The inclusion of antibodies to LIF served as a true control and in this regard as an irrelevant antibody. The absent effect of anti-LIF is therefore expected.

The major producer of both IL-5 and RANTES is probably the T lymphocyte.³¹ However, IL-5 was shown to be produced also by mast cells and the eosinophils themselves,^{32,33} and RANTES by

many other cells, such as epithelial cells.³⁴ In a recent study we found that the presence of eosinophil chemotactic activity was correlated to the number of CD45RA T lymphocytes.²³ Other studies have shown that IL-5 is predominantly produced by the T_H2-like cell. It is obvious that the interpretation of correlations between activities and cells should be made with caution, because the activity in the BALF is determined by at least two molecules.

In conclusion, we have in this preliminary study shown that IL-5 and RANTES are most likely two of the major chemoattractants in the lungs of patients with allergic asthma. In this respect, IL-5 seems to act as a necessary cofactor to true chemotactic factors, such as RANTES. Our findings add to other findings of the importance and central role of IL-5 for the inflammatory process underlying asthma.

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