

Eosinophil recruitment is associated with IL-5, but not with RANTES, twenty-four hours after allergen challenge

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Several lines of evidence suggest that the chemokine RANTES may play a role in eosinophilia observed during allergic inflammation. To test this hypothesis, six patients with allergic asthma were studied. After performing bronchoalveolar lavage in a lung segment (baseline), segmental bronchoprovocation was performed with saline solution in another segment and with ragweed in a third segment. Bronchoalveolar lavage was performed 24 hours later in the saline-challenged (sham) and ragweed-challenged lung segments. The bronchoalveolar lavage fluids from the baseline, sham, and ragweed segments were analyzed for cell counts and for the levels of IL-5, RANTES, and eosinophil-derived neurotoxin. IL-5 levels were elevated in the ragweed (984 ± 588 pg/ml) compared with sham segments (2.8 ± 0.2 pg/ml, $p = 0.02$). Likewise, RANTES levels were elevated in the ragweed (12.93 ± 3.4 pg/ml) compared with the sham segments (3.05 ± 1.19 pg/ml, $p = 0.006$). The IL-5 levels correlated with both eosinophil numbers ($r = 0.90$, $p < 0.02$) and eosinophil-derived neurotoxin levels ($r = 0.89$, $p < 0.02$). In contrast, RANTES levels did not correlate with either eosinophil numbers or eosinophil-derived neurotoxin levels. These results indicate that although both IL-5 and RANTES are elevated 24 hours after allergen challenge, only IL-5 correlates with eosinophil recruitment and degranulation. (*J Allergy Clin Immunol* 1996;97:1272-8.)

Key words: IL-5, RANTES, asthma, chemokines, cytokines, late phase reaction, eosinophils, EDN

Eosinophil recruitment and degranulation are thought to be important in the pathogenesis of asthma.¹⁻³ Segmental bronchoprovocation (SBP) with allergen has been performed by various investigators to study recruitment of eosinophils to the airways in patients with allergic rhinitis and asth-

Abbreviations used

BAL: Bronchoalveolar lavage
EDN: Eosinophil-derived neurotoxin
SBP: Segmental bronchoprovocation

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ma.⁴⁻⁸ We have previously shown that IL-5 is associated with eosinophil recruitment and degranulation in patients with allergic rhinitis after SBP with allergen.⁷⁻⁸ Several lines of evidence suggest that RANTES, a member of the 8 kd C-C group of chemokines, may also play an important role in eosinophil recruitment and degranulation during allergic inflammation.⁹⁻¹⁰ First, in vitro and in vivo studies suggest a pathway of eosinophil recruitment involving RANTES.¹¹⁻¹⁶ Second, RANTES induces eosinophil cationic protein release from eosinophils.¹⁴ Third, because RANTES is chemotactic for CD4 memory T cells, it may play a role in

TABLE I. Patient summary

Patient No.	Age (yr)	Sex	SPT Response		FEV ₁ (% predicted)	SBP dose (PNU‡)
			Immediate*	Late†		
1	18	M	11	+	92	230
2	42	F	5.5	+	103	500
3	31	M	6	+	90	500
4	20	F	7	+	87	500
5	35	M	12.5	+	80	500
6	39	M	12	+	81	230

SPT, Skin prick test; PNU, protein nitrogen units.

*Wheal in millimeters after 15 minutes.

†Presence of > 10 mm cutaneous induration after 5 to 9 hours.

‡Dose of ragweed allergen used for SBP.

late-phase inflammation of the airways by attracting IL-5-producing T_{H2} cells to the airways.^{11, 17-20}

Little is known about the presence or significance of RANTES in allergic inflammation that occurs in patients with asthma. In this study, SBP was performed with both saline solution and ragweed allergen in six patients with asthma. Bronchoalveolar lavage (BAL) was performed in challenged lung segments from these patients 24 hours later, and the BAL fluids were analyzed for the presence of IL-5 and RANTES by means of immunoenzymetric assays. The results of this study indicate that IL-5 and RANTES levels are elevated in the BAL fluids during allergic inflammation of the lungs in patients with asthma. Unexpectedly, eosinophil recruitment after allergen challenge was only associated with IL-5 and not with RANTES.

METHODS

Patients

Six patients with mild asthma volunteered for this study. The patients had a history of exacerbation of symptoms of allergic rhinitis and asthma during the ragweed season. All patients met the following inclusion criteria: (1) an immediate wheal and flare response to ragweed extract at a 1:10 wt/vol dilution, (2) a 10 mm or greater late-phase cutaneous induration 5 to 9 hours after this skin prick test, (3) absence of symptoms at the time of the study, (4) FEV₁ greater than 75% of predicted value, (5) absence of upper respiratory tract infection for at least 2 months before SBP, and (6) no need for either systemic or topical glucocorticosteroids, cromolyn sodium, or nedocromil for at least 2 months before SBP. Table I summarizes the patient data. This study was approved by the Mayo Clinic Institutional Review Board. All patients gave written informed consent to participate in the study.

Study design

The patients underwent BAL in one lung segment ("baseline" BAL fluid), followed by SBP with saline solution in another segment and SBP with ragweed extract in a third segment. In this way, each patient served as his or her own control. The ragweed extract used in this study (Greer Laboratories, Inc., Lenoir, N.C.) had less than 12.5 endotoxin units per milliliter; we have previously shown that this amount of endotoxin does not alter recruitment of cells (i.e., increase recruitment of neutrophils and reduce recruitment of eosinophils) to the challenged segments after SBP with allergen.²¹ A BAL was performed in these challenged segments 24 hours later. The BAL fluids from the segments challenged with saline solution and ragweed were termed *sham* and *ragweed*, respectively.

Bronchoscopy, BAL, and SBP

This procedure was similar to that described previously.²¹ Briefly, all patients were admitted to the clinical research unit at the Mayo Clinic. The procedure was performed with an Olympus PD20 bronchoscope (Olympus America, Inc., Success, N.Y.) by the oral route. Topical anesthetic agents were benzocaine (20%) and lidocaine (2%). Intravenous midazolam was given as needed to control anxiety and reduce cough. The bronchoscope was advanced to a subsegmental level until it could not be advanced any further, and from this "wedged" position, BAL or SBP was performed. A BAL was performed with four 25 ml aliquots of prewarmed (37°C) normal saline solution in one segment. SBP was performed by instillation of 5 ml of sterile saline solution into another segment and ragweed allergen dissolved in 5 ml of sterile saline solution into a third segment. The method of selecting the dose of ragweed allergen was a modification of the method used by Wenzel et al.²² and has been described earlier.^{21, 22} BAL was performed 24 hours later in the two segments in which SBP had been performed.

TABLE II. Mean $\pm$ SEM of the differential cell count in BAL cells

	Baseline	Sham	Ragweed	<i>p</i> Value 1*	<i>p</i> Value 2†	<i>p</i> Value 3‡
Eosinophils	0.62 $\pm$ 0.3	0.65 $\pm$ 0.2	34.86 $\pm$ 13.4	NS	0.005	0.005
Neutrophils	6.54 $\pm$ 4.5	15.04 $\pm$ 6.3	15.24 $\pm$ 2.7	NS	NS	NS
Lymphocytes	13.79 $\pm$ 3.5	10.66 $\pm$ 2.5	10.56 $\pm$ 1.6	NS	NS	NS
Macrophages	78.56 $\pm$ 6.2	73.1 $\pm$ 7.9	38.73 $\pm$ 12.6	NS	NS	NS

All cell counts are expressed as percentage of total BAL cells.

NS, Not significant.

*Sham versus baseline.

†Ragweed versus baseline.

‡Ragweed versus sham.

BAL processing

The processing of BAL fluids was similar to a protocol described earlier.²¹ Briefly, the BAL fluids were passed through nylon mesh to remove mucus and centrifuged at 200 *g* to pellet the BAL cells. The supernatant BAL fluid without cells was frozen at -20°C for later analyses. The pelleted BAL cells were resuspended, and total cell counts were determined by counting in a hemocytometer. For performing differential counts, cytocentrifuge preparations were stained with Diff Quick (Baxter Healthcare Corp., Miami, Fla.); and the eosinophils, neutrophils, lymphocytes, and macrophages in the stained slides were enumerated by counting at least 400 cells.

Measurement of IL-5 and RANTES in BAL fluids

The frozen BAL fluids without cells were thawed and analyzed for IL-5 and RANTES. An immunoradiometric assay was used to quantify IL-5 levels in BAL fluids as previously described.²³ Initially, assays were performed on unconcentrated BAL fluids. The lower limit of detection for this assay was 13 pg/ml. If IL-5 was not detected in unconcentrated BAL fluids, the BAL fluids were concentrated fivefold and reanalyzed. If IL-5 was not detected even after fivefold concentration, a value of 2.6 pg/ml (equal to the lower limit of detection, 13 pg/ml, divided by 5 to account for the fivefold concentration of BAL fluids) was arbitrarily assigned to it.

A commercial immunoenzymetric assay kit was used to quantify RANTES in the BAL fluids (R & D Systems, Minneapolis, Minn.). Initially, assays were performed in unconcentrated BAL fluids. The lower limit of detection for this assay was 8 pg/ml. According to the manufacturers, the kit exhibits no cross-reactivity with other cytokines. If RANTES was not detected in unconcentrated BAL fluids, the BAL fluids were concentrated fivefold and reanalyzed. If RANTES was not detected even after fivefold concentration, a value of 1.6 pg/ml (equal to the lower limit of detection, 8 pg/ml, divided by 5 to account for the fivefold concentration of BAL fluids) was arbitrarily assigned to it.

Measurement of EDN in BAL fluids

Eosinophil-derived neurotoxin (EDN) was measured by a competitive binding radioimmunoassay with a polyclonal antibody to EDN as previously described.⁴ The sensitivity of this assay was 2 ng/ml.

Statistics

To determine whether allergen challenge resulted in significant recruitment of eosinophils and elevation of IL-5, RANTES, and EDN levels in the BAL fluids, the log-transformed values from baseline, sham, and ragweed segments were analyzed by analysis of variance. To compare pairs of treatments, the Bonferroni procedure was used in conjunction with significant analyses of variance. Pearson correlations were determined between BAL variables in two different ways. First, correlations were determined between variables in the ragweed segment alone. Second, the difference between ragweed and sham segments was calculated for IL-5 levels ($\Delta \text{IL-5}_{\text{ragweed-sham}}$), RANTES levels ($\Delta \text{RANTES}_{\text{ragweed-sham}}$), EDN levels ($\Delta \text{EDN}_{\text{ragweed-sham}}$), and the total number of each cell type (e.g., $\Delta \text{total eosinophils}_{\text{ragweed-sham}}$); Pearson correlations were computed for these differences (Δ).

RESULTS

The BAL differential cell counts are summarized in Table II. The ragweed segment had a higher percentage of eosinophils compared with baseline and sham segments. In contrast, the percentages of neutrophils, lymphocytes, and macrophages in the ragweed segment were not different from either baseline or sham.

The total numbers of each cell type in the BAL fluids are summarized in Table III. The total number of eosinophils was higher in the ragweed segment than in the baseline ($p = 0.002$) and sham segments ($p = 0.006$). These data indicate that eosinophils were recruited 24 hours after allergen challenge. The total number of neutrophils was

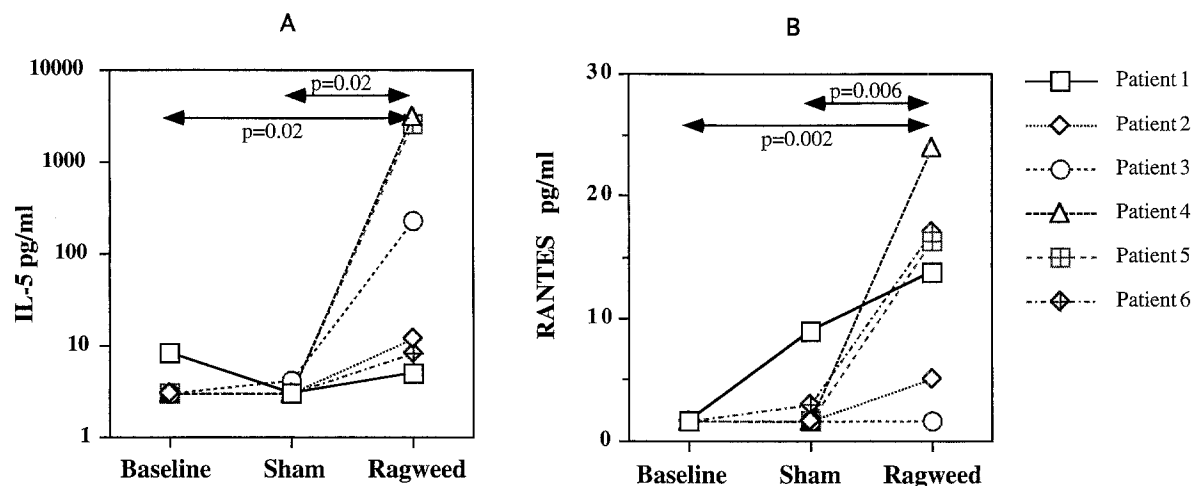


FIG. 1. IL-5 and RANTES levels in BAL fluids. BAL fluids from baseline, sham, and ragweed segments were analyzed for IL-5 (**panel A**) and RANTES (**panel B**) by immunoradiometric (IL-5) and immunoenzymetric (RANTES) assays. The lower limits of detection for the IL-5 and RANTES assays were 13 pg/ml and 8 pg/ml, respectively. If the IL-5 and RANTES levels were below limits of detection, the fluids were concentrated fivefold and reanalyzed. If IL-5 was not detected in these fivefold-concentrated BAL fluids, a value of 2.6 pg/ml (equal to the lower limit of detection, 13 pg/ml, divided by 5 to account for fivefold concentration of BAL fluids) was assigned to it. Likewise, if RANTES was not detected in these fivefold-concentrated BAL fluids, a value of 1.6 pg/ml (equal to the lower limit of detection, 8 pg/ml, divided by 5 to account for fivefold concentration of BAL fluids) was assigned to it. All values in both panels are expressed as picograms per milliliter in unconcentrated BAL fluids.

TABLE III. Mean $\pm$ SEM of the total numbers of each cell type $\times 10^6$ in BAL fluids

	Baseline	Sham	Ragweed	p Value 1*	p Value 2†	p Value 3‡
Eosinophils	0.06 $\pm$ 0.03	0.09 $\pm$ 0.03	6.67 $\pm$ 3.24	NS	0.002	0.006
Neutrophils	0.33 $\pm$ 0.09	4.92 $\pm$ 3.05	1.90 $\pm$ 0.42	NS	0.012	NS
Lymphocytes	1.97 $\pm$ 1.07	2.57 $\pm$ 1.27	1.73 $\pm$ 0.64	NS	NS	NS
Macrophages	9.65 $\pm$ 2.93	11.39 $\pm$ 3.27	3.47 $\pm$ 0.95	NS	NS	NS

NS, Not significant.

*Sham versus baseline.

†Ragweed versus baseline.

‡Ragweed versus sham.

higher in the ragweed segment compared with baseline segment ($p = 0.012$), but not sham segment ($p = \text{NS}$). It is therefore likely that the higher number of neutrophils in the ragweed segment compared with baseline was due to the procedure itself (i.e., SBP followed by BAL) and was not an effect of ragweed allergen. The numbers of lymphocytes and macrophages in the ragweed segment were not different from those in baseline or sham segments.

Fig. 1 shows the results of the IL-5 and RANTES assays performed in the BAL fluids of individual patients. IL-5 levels were elevated in the BAL fluids obtained from the ragweed segment (984 ± 588 pg/ml), compared with each of

the levels in the baseline (3.5 ± 0.9 pg/ml, $p = 0.02$) and sham segments (2.8 ± 0.2 pg/ml, $p = 0.02$). RANTES levels were also elevated in the BAL fluids obtained from the ragweed segment (12.93 ± 3.4 pg/ml), compared with each of the levels in the baseline (1.6 ± 0 pg/ml, $p = 0.002$) and sham segments (3.05 ± 1.19 pg/ml, $p = 0.006$). Likewise, EDN levels were elevated in the BAL fluids obtained from the ragweed segment (37.75 ± 18.96 ng/ml), compared with each of the levels in the baseline (2.17 ± 0.09 ng/ml, $p = 0.02$) and sham segments (2.23 ± 0.15 ng/ml, $p = 0.02$).

Fig. 2 shows the correlation of IL-5 and RANTES with eosinophil numbers and EDN levels in the BAL

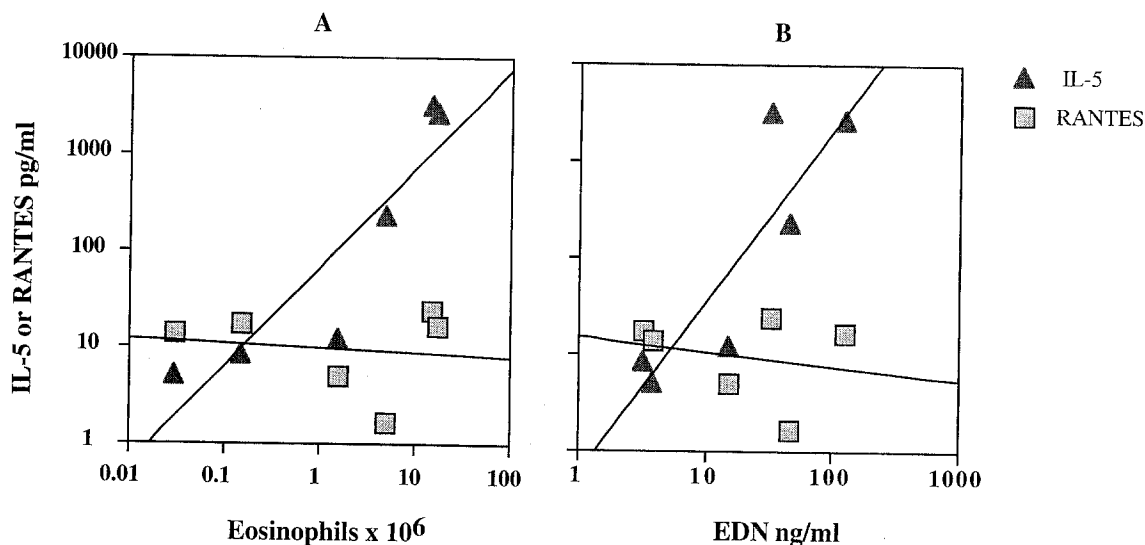


FIG. 2. Correlation of IL-5 and RANTES levels with eosinophil numbers and EDN levels in the ragweed segment. **Panel A** shows that IL-5 levels correlated with eosinophil numbers ($r = 0.91$, $p < 0.01$). In contrast, RANTES levels did not correlate with eosinophil numbers ($r = 0.11$, $p = 0.83$). Likewise, **panel B** shows that IL-5 levels correlated with EDN levels ($r = 0.89$, $p < 0.02$). In contrast, RANTES levels did not correlate with EDN levels ($r = 0.21$, $p = 0.69$).

TABLE IV. Pearson correlations performed on the Δ ragweed-sham values

	IL-5 (pg/ml)		RANTES (pg/ml)	
	r Value	p value	r Value	p value
Eosinophils $\times 10^6$	0.90	<0.02	0.40	0.44
Neutrophils $\times 10^6$	0.75	0.09	0.21	0.70
Lymphocytes $\times 10^6$	0.80	0.06	0.34	0.51
Macrophages $\times 10^6$	-0.62	0.19	0.52	0.29
EDN (ng/ml)	0.89	<0.02	0.31	0.55
IL-5 (pg/ml)	—	—	0.62	0.19
RANTES (pg/ml)	0.62	0.19	—	—

fluids. IL-5 levels in the ragweed segment correlated with the total numbers of eosinophils in that segment ($r = 0.91$, $p < 0.01$). Similar correlations were obtained when Δ IL-5_{ragweed-sham} was analyzed with Δ total eosinophils_{ragweed-sham} ($r = 0.90$, $p < 0.02$). Thus both methods of statistical analyses indicate that IL-5 levels correlate with eosinophil numbers. Likewise, both methods of statistical analyses indicate that IL-5 levels correlate with EDN levels ($r = 0.89$, $p < 0.02$). In contrast, neither method of statistical analysis demonstrates significant correlations between RANTES levels and either the total number of eosinophils or the EDN levels. For example, a correlation performed between Δ RANTES_{ragweed-sham} and the Δ total eosinophils_{ragweed-sham} yielded

$r = 0.40$, $p = 0.44$. Further, a correlation performed between Δ RANTES_{ragweed-sham} and the Δ EDN_{ragweed-sham} yielded $r = 0.31$, $p = 0.55$. Finally, neither IL-5 nor RANTES levels correlated with neutrophil, lymphocyte, or macrophage numbers (Table IV).

DISCUSSION

In this study, RANTES levels were elevated in the BAL fluids of patients with asthma after SBP with ragweed. RANTES and IL-8 are members of a family of immunoregulatory molecules known as chemokines. The characteristic feature of chemokines is the conservation of four cysteine residues.²⁴ These chemokines are divided into two groups, the C-X-C and the C-C, on the basis of the presence or absence of an intervening amino acid between the two cysteines.²⁴ RANTES is a member of the family of C-C chemokines. The RANTES gene is located in human chromosome 17q11.2-q12.²⁵ RANTES is released from platelets and produced by a variety of cells including T cells, fibroblasts, monocytes, delayed-type hypersensitivity granuloma cells, and other cells.^{13, 26, 27} RANTES and the related chemokines, macrophage chemotactic protein-1, macrophage inflammatory protein-1 α , and macrophage inflammatory protein-1 β , bind to a common G-protein-linked C-C chemokine receptor, C-C CKR-1.^{24, 28, 29}

Prior studies have shown that RANTES is a

chemoattractant for human eosinophils.¹¹⁻¹⁵ These observations have led investigators to speculate that RANTES plays a role in allergen-induced eosinophil recruitment.^{9,10} Unexpectedly, when correlations were performed with standard statistical methods in this study, the number of eosinophils correlated with IL-5, but not with RANTES. Further, EDN levels correlated with IL-5, but not with RANTES. We have previously shown that IL-5 is associated with eosinophil recruitment in patients with allergic rhinitis 24 hours after SBP with allergen.^{7,8} Sedgwick et al.⁴ have also shown that IL-5 levels in the BAL fluids of patients with allergic rhinitis correlated with the number of eosinophils recruited 48 hours after allergen challenge. The results of this study extend the previously described association of IL-5 and eosinophils in patients with allergic rhinitis to patients with asthma. One explanation for the lack of correlation between RANTES and eosinophil numbers is that RANTES does not play a role in allergen-induced eosinophil recruitment. However, a role for RANTES in eosinophil recruitment cannot be ruled out because RANTES may act by enhancing recruitment of IL-5-primed eosinophils.³⁰

There are at least two explanations for the correlation of eosinophil numbers with IL-5, but not with RANTES, in this study. First, IL-5 and RANTES may be produced by different cell types. Previous studies have shown that IL-5 is produced by T cells,¹⁷ eosinophils,³¹⁻³³ and mast cells.³⁴ In contrast, RANTES is present in platelets,¹³ and the RANTES messenger RNA is expressed in T cells,³⁵ monocytes, and other cell types.²⁶ Second, IL-5 and RANTES may be produced at different times and in different quantities after allergen challenge. For example, the levels of IL-5 detected after nasal allergen challenge in patients with seasonal allergic rhinitis follow a biphasic early- and late-phase pattern in some patients or a monophasic late-phase pattern in other patients.³⁶ However, the kinetics of production of RANTES after allergen challenge in atopic patients has not been described.

The RANTES levels in the BAL fluids were low in this study, that is, below 30 pg/ml. Although chemokines are generally thought of as soluble mediators, a growing body of evidence suggests that RANTES and other chemokines interact with glycosaminoglycans, such as heparinoids, in the intercellular matrix.^{37,38} It has been proposed that matrix-bound RANTES and other chemokines act as haptotactic and chemotactic mediators of inflammatory cell adhesion and migration.^{37,39} The

low levels of RANTES in the BAL fluids suggest that RANTES is predominantly anchored to the intercellular matrix and only a small fraction of it is soluble; this would also explain the lack of correlation between soluble RANTES levels in BAL fluids and eosinophil numbers. The importance of RANTES may relate to its ability to localize eosinophils within airways (e.g., basement membrane of epithelium), rather than being primarily responsible for recruitment from blood vessels into the tissues. Finally, the low levels of RANTES in BAL fluids could be derived from platelet degranulation occurring during SBP with allergen, rather than antigen-induced production by either infiltrating cells (e.g., T cells) or resident cells (e.g., epithelial cells).

In summary, our results indicate that although both IL-5 and RANTES are elevated 24 hours after SBP with ragweed allergen, only IL-5 is correlated with eosinophil recruitment and degranulation. The role of RANTES in allergic inflammation needs to be defined.

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