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Eotaxin and monocyte chemoattractant protein-1 in chronic eosinophilic pneumonia

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ABSTRACT: Chronic eosinophilic pneumonia (CEP) is characterized by chronic or recurrent pulmonary infiltrates with eosinophils, but the precise mechanism of eosinophil accumulation has not been fully elucidated. Eotaxin is one of the CC chemokines that selectively recruits eosinophils and contributes to the pathogenesis of allergic airway diseases including asthma, but its roles in pathogenesis of CEP have not been fully elucidated.

The authors measured concentrations of eotaxin and other CC chemokines, monocyte chemoattractant protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted, macrophage inflammatory protein-1α, and the eosinophil activating Th2 cytokine interleukin (IL)-5 in bronchoalveolar lavage (BAL) fluid from CEP patients (n = 11), and compared these concentrations with those from control subjects (n = 6).

The eotaxin (904 ± 203 *versus* 29 ± 7 pg·mL^{-1}, p = 0.0001), MCP-1 (194 ± 57 *versus* 15 ± 2 pg·mL^{-1}, p < 0.05), and IL-5 (7.8 ± 2.0 *versus* 2.7 ± 0.6 pg·mL^{-1}, p < 0.05) levels were significantly higher for cases with CEP in comparison to those serving as controls. Proportions of eosinophil and lymphocyte counts were greater in BAL fluid from CEP patients. Eotaxin and IL-5 levels correlated with the proportion of eosinophils in BAL fluid from CEP patients. MCP-1 correlated with the relative lymphocyte numbers.

In short, eotaxin, interleukin-5, and monocyte chemoattractant protein-1 levels were higher in the BAL fluid of CEP patients and these levels may contribute to eosinophil and lymphocyte recruitment and activation in the airways as found with this disorder. *Eur Respir J 2001; 17: 962–968.*

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Chronic eosinophilic pneumonia (CEP) is an idiopathic pulmonary disorder characterized by marked eosinophil accumulation in the airways. Patients with CEP have chronic clinical symptoms including fever, night sweats, and dyspnoea and ~50% of the patients experience asthmatic symptoms. The prognosis is usually good as the radiographic infiltrates and symptoms are usually reduced by treatment with corticosteroids, but relapse is not unusual during cessation of corticosteroid treatment [1–3].

Eotaxin is one of the CC chemokines that selectively recruits eosinophils to tissues and has recently been reported to contribute to the pathogenesis of allergic airway inflammation including bronchial asthma [4–8]. Previous studies demonstrated that eotaxin and interleukin (IL)-5, the potent eosinophil-activating Th2 cytokine, collaborate to promote eosinophil recruitment into inflamed tissues in animal models [9]. In addition, increased production of various CC chemokines like eotaxin, regulated upon activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α, and monocyte chemoattractant protein (MCP)-1 has been demonstrated in allergic airway inflammation with eosinophils [10–12].

Since eosinophil accumulation is much more prominent in the lung parenchyma of CEP patients compared to those in asthmatics, CC chemokines active on eosinophils may contribute to the processes of eosinophil mobilization in CEP. KURASHIMA *et al.* [13] reported that the CC chemokine, RANTES, had higher levels in bronchoalveolar lavage (BAL) fluid obtained from CEP patients, but the roles of the eosinophil-selective CC chemokine eotaxin in CEP have not been fully elucidated. To determine whether eotaxin plays a significant role in the pathogenesis of CEP coordinating with other CC chemokines and IL-5, concentrations of eotaxin, RANTES, MIP-1α, MCP-1, and IL-5 were measured in BAL fluid harvested from CEP patients, and compared to those obtained from healthy control subjects and to those from patients with other interstitial lung diseases.

Methods

Study populations

Eleven patients with CEP (19–75-yr-olds, male/female (M/F): 9/2, current smoker/exsmoker/nonsmoker

(C/E/N) 2/6/3) were admitted to Keio University Hospital between 1991–1998. Inclusion criteria were patients with: 1) clinical symptoms persisting for >2 weeks (fever, weight loss, dry cough, and dyspnoea); 2) predominantly peripheral pulmonary infiltrates on chest radiographs; 3) increased eosinophil counts in BAL fluid (proportion of eosinophils >10%); and 4) the absence of predisposing causes such as a drug allergy, a parasite infection, vasculitis, or inhaled antigens [1–3, 13]. No CEP patients had been treated with either corticosteroids or immunosuppressive agents when BAL was performed. Transbronchial lung biopsy (TBLB) was performed on eight patients with CEP and the pathological findings were consistent with CEP, while vasculitis, granuloma, and significant fibrosis were not observed. Nine patients with idiopathic pulmonary fibrosis (IPF) (37–75 yrs, M/F 7/2, C/E/N 2/4/3) and eight with sarcoidosis (SAR) (24–47 yrs, M/F 4/4, C/E/N 4/1/3) were evaluated as disease controls in the current study. These patients were admitted to Keio University Hospital between 1997–1998. The IPF patients fulfilled all of the major diagnostic criteria and at least three of the four minor criteria described in the international consensus statement [14]. TBLB specimens obtained from the IPF patients suggested pulmonary fibrosis but had no features supporting other diseases. The SAR patients had abnormal findings on chest radiographs (stage I or II) and were diagnosed by TBLB specimens showing noncaseating epithelioid cell granulomas [15]. None of them had any history of inhaling inorganic materials known to cause granulomatous diseases. Six control subjects (CTL) (18–25 yrs, M/F 6/0, C/E/N 0/0/6) were healthy volunteers and exhibited neither respiratory symptoms nor abnormal chest radiograph findings. None of the subjects in the IPF, SAR, and CTL groups had been treated with either corticosteroids or immunosuppressive agents before the BAL procedures. Samples were collected after obtaining informed consent from each subject. The study was approved by the ethics committee at Keio University Hospital.

Bronchoalveolar lavage

BAL was performed in the involved lung segment or middle lobe. The bronchoscope was advanced into the involved segment and wedged; sterile normal saline was instilled three times in 50-mL aliquots. The recovered BAL fluid was centrifuged at $200 \times g$ and 4°C for 5 min, and cell-free supernatants were collected and frozen at -70°C until use. The cell pellets were used for the differential counts on Wright-Giemsa-stained preparations.

Measurement of chemokine and interleukin-5 concentrations

Concentrations of eotaxin in BAL fluids were measured by enzyme linked immunosorbent assay (ELISA) in duplicate as previously described [4, 5, 7, 8], with a detection limit of 15.6 pg·mL^{-1}. The concentrations of MCP-1, RANTES, IL-5, and MIP-1α were measured in duplicate using ELISA kits from R&D Systems (Minneapolis, MN, USA). The detection limits were 5.0, 5.0, 3.0, and 7.0 pg·mL^{-1}, respectively. All measurements were performed using unconcentrated BAL fluids.

Statistical analysis

Data were expressed as mean±SEM. Analysis of variance was used to compare mean values. Simple linear regression analysis was performed to evaluate the correlation between concentrations of each chemokine or IL-5 in BAL supernatants and proportions of BAL cells. In addition, normalized multiple linear regression analysis was employed to assess the correlation between the various mediator concentrations and the BAL inflammatory cells. A p-value <0.05 was considered statistically significant.

Results

Characteristics of clinical manifestations in chronic eosinophilic pneumonia patients

Clinical features of CEP patients are summarized in table 1. Patient age ranged 19–75 yrs and the duration of symptoms before diagnosis ranged 2–36 weeks. The chronic, progressive illness did not improve after treatment with antibiotics for any of the patients, but significantly improved after corticosteroid treatment for eight patients. For three patients, the symptoms gradually lessened without the administration of steroids. The reason for this choice, *i.e.* no steroid treatment, was hyperglycaemia in one patient (case 9) and slow but spontaneous regression in two patients (cases 1 and 4). Previous asthmatic symptoms were confirmed for five patients. Eosinophil percentages of BAL fluids ranged 14.3–71.1%. A peripheral blood eosinophilia level of >6% was seen in eight cases.

Bronchoalveolar cell differentials

Total cell counts of BAL fluid from CEP patients were higher than those from the CTL group, but not in comparison with IPF and SAR (CEP: 11.6±3.5 *versus* CTL: $2.7 \pm 0.5 \times 10^5$ mL^{-1}, $p<0.05$, IPF: $5.9 \pm 1.5 \times 10^5$ mL^{-1}, SAR: $6.5 \pm 1.0 \times 10^5$ mL^{-1}). The proportion of eosinophils and lymphocytes in BAL fluid obtained from CEP, IPF, SAR, and CTL is shown in figure 1. The eosinophil ratio was markedly higher in patients with CEP. The lymphocyte percentage was the highest for SAR and the second highest for CEP. The percentage of neutrophils in BAL fluid was the highest for IPF patients (CEP: 3.7±1.0, IPF: 5.6±2.1, SAR: 0.7±0.3, CTL: 0.3±0.1%, $p<0.05$, IPF *versus* SAR or CTL).

Chemokine and interleukin-5 concentrations in bronchoalveolar lavage fluids

Eotaxin recovery was markedly augmented in the CEP group (904±203 pg·mL^{-1}) than in other groups

Table 1. – Patient profiles for chronic eosinophilic pneumonia

Case	Sex	Age yrs	Duration* weeks	Asthma	Relapse	BALF TCC $\times 10^5 \cdot ml^{-1}$	BALF Eo%	PB Eo%
1	M	37	8	+	+	5.9	14.3	24.0
2	M	50	28	-	+	14.2	18.3	3.3
3	M	73	8	-	-	5.6	62.1	19.6
4	M	19	2	-	-	16.3	71.1	12.0
5	M	58	8	+	-	5.8	36.5	33.1
6	M	70	12	+	-	6.2	20.3	11.9
7	M	52	12	+	-	10.2	22.6	36.8
8	M	62	36	-	+	2.8	37.6	39.7
9#	M	75	4	-	-	7.1	27.3	18.4
10	F	39	28	+	+	8.8	29.1	3.8
11#	F	57	3	-	-	44.4	50.0	2.0
Mean ± SEM		53.8 ± 5.1	12.7 ± 3.2			11.6 ± 3.5	35.5 ± 5.6	18.6 ± 4.0

M: male; F: female; BALF: bronchoalveolar lavage fluid; TCC: total cell count; Eo%: percentage of eosinophils; PB: peripheral blood; #: patients with eotaxin levels lower than 30 $pg \cdot ml^{-1}$; *: weeks before diagnosis.

(CTL: 29 ± 7, IPF: 161 ± 17, SAR: 149 ± 21 $pg \cdot mL^{-1}$) (fig. 2a). In two CEP patients, BAL eotaxin levels were <30 $pg \cdot mL^{-1}$ ($\sim$ the mean value in the CTL group) despite the presence of eosinophil accumulation (fig. 2a). In contrast, MCP-1 levels were higher for the IPF (354 ± 69 $pg \cdot mL^{-1}$) and CEP (194 ± 57 $pg \cdot mL^{-1}$) groups than for the CTL group (15 ± 2 $pg \cdot mL^{-1}$) (fig. 2b). Although the concentrations of RANTES and MIP-1α were lower than those of eotaxin and MCP-1 for CEP patients and no statistical difference was observed in the levels of both chemokines between CEP and CTL, their levels in the CEP group tended to be somewhat higher than those in the CTL group (RANTES: 30 ± 14 *versus* 1.2 ± 0.6 $pg \cdot mL^{-1}$, $p=0.052$, MIP-1α: 31 ± 13 *versus* 11 ± 0.2, $p=0.13$) (figs. 2c and 2d). The IL-5 levels detected in BAL fluid were relatively low, but they were significantly higher in the CEP group (7.8 ± 2.0 $pg \cdot mL^{-1}$) than in the SAR (3.0 ± 0.2 $pg \cdot mL^{-1}$) and CTL (2.7 ± 0.6) groups (fig. 2e).

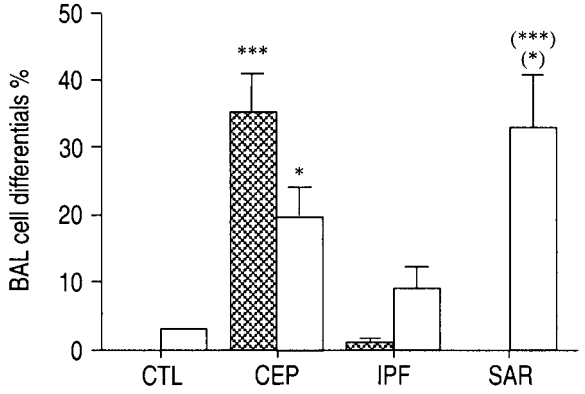

Fig. 1. – Percentage of eosinophils (▩) and lymphocytes (□) in bronchoalveolar lavage (BAL) fluid. Data represents the mean ± SEM. CTL: control subjects (n = 6), CEP: chronic eosinophilic pneumonia (n = 11), IPF: idiopathic pulmonary fibrosis (n = 9); SAR: sarcoidosis (n = 8). ***: $p<0.001$ *versus* the other three groups, (***): $p<0.001$ *versus* CTL; (*): $p<0.05$ *versus* CEP; *: $p<0.05$ *versus* CTL.

Correlation between the chemokine and interleukin-5 concentrations and bronchoalveolar eosinophils and lymphocytes

In order to elucidate the contribution of chemokines and IL-5 to leukocyte recruitment to the airspaces in CEP, correlation of the concentrations of these mediators with the percentage of eosinophils or lymphocytes in BAL fluid was evaluated using the data in CEP patients and CTL subjects. Eotaxin and IL-5 levels significantly correlated with the percentage of eosinophils by simple linear regression analysis (figs. 3a and 3b). MCP-1 distinctly correlated with relative lymphocyte numbers (fig. 3c). The correlation coefficients (r) and p-values between the other combinations in simple linear regression analysis are presented below. Eosinophil *versus* MCP-1: $r=0.34$ ($p=0.18$), MIP-1α: $r=0.27$ ($p=0.29$), RANTES: $r=0.20$ ($p=0.44$). Lymphocyte *versus* eotaxin: $r=0.11$ ($p=0.68$), MIP-1α: $r=0.03$ ($p=0.90$), RANTES: $r=0.15$ ($p=0.57$), IL-5: $r=0.10$ ($p=0.70$).

In multiple linear regression analysis, the coefficient of determination (R^2) between the relative eosinophil numbers and the concentrations of the five mediators in BAL fluid was 0.70 ($p=0.01$). Judging from the standard partial regression coefficients (SPRC), IL-5 and eotaxin levels had the dominant explanatory power for the relative eosinophil numbers in the BAL fluid, while those of the other three substances (MCP-1, MIP-1α, and RANTES) did not (table 2). The R^2 value between the relative lymphocyte numbers and the concentrations of the five mediators in the BAL fluid was 0.71 ($p=0.01$). Only the SPRC observed for MCP-1 was statistically significant (table 2). The results obtained from multiple linear regression analysis were qualitatively consistent with those from simple linear regression analysis.

Discussion

CEP is an idiopathic pulmonary disease characterized by marked lung parenchyma eosinophilia. Based on the selective effects of eotaxin on eosinophils and

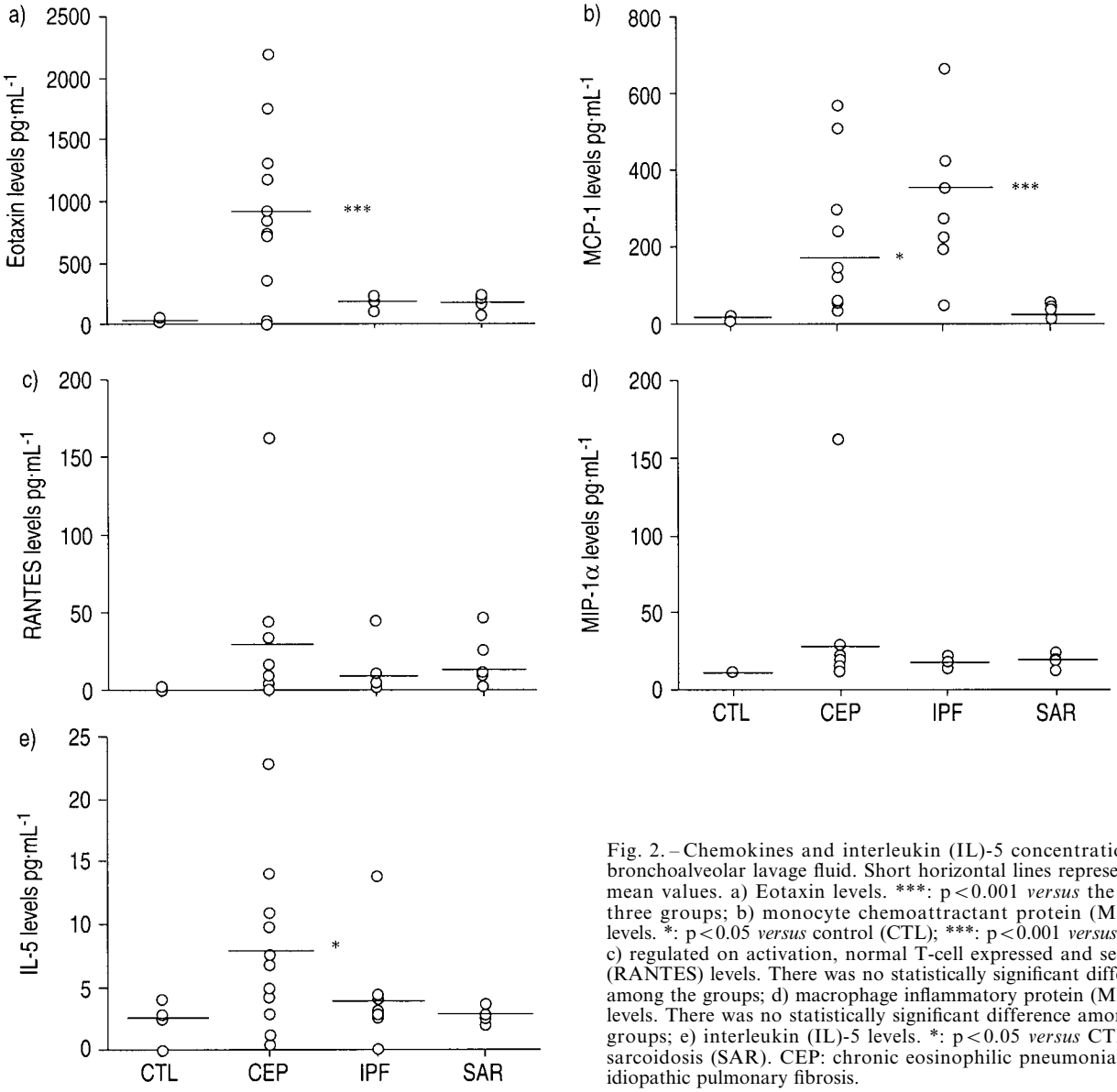

Fig. 2. – Chemokines and interleukin (IL)-5 concentrations in bronchoalveolar lavage fluid. Short horizontal lines represent the mean values. a) Eotaxin levels. ***: $p<0.001$ *versus* the other three groups; b) monocyte chemoattractant protein (MCP)-1 levels. *: $p<0.05$ *versus* control (CTL); ***: $p<0.001$ *versus* CTL; c) regulated on activation, normal T-cell expressed and secreted (RANTES) levels. There was no statistically significant difference among the groups; d) macrophage inflammatory protein (MIP)-1α levels. There was no statistically significant difference among the groups; e) interleukin (IL)-5 levels. *: $p<0.05$ *versus* CTL and sarcoidosis (SAR). CEP: chronic eosinophilic pneumonia; IPF: idiopathic pulmonary fibrosis.

prominent eosinophil accumulation in CEP patients, the authors hypothesized that eotaxin is involved in the pathogenesis of CEP. Results suggest that eotaxin levels markedly increase and significantly correlate with the proportion of BAL eosinophils for the CEP patients, and the coordinated upregulation of eotaxin, IL-5, and MCP-1 may contribute to eosinophil and lymphocyte mobilization as observed in allergic airway inflammation including bronchial asthma [9–12].

Although ~50% of CEP patients experience asthmatic symptoms, no specific allergens have been detected in CEP patients. In addition, BAL eosinophilia is not as prominent in asthma as it is in CEP (median; 3.9%) [4] and eosinophil-related pulmonary infiltrates are not observed in asthmatics. Only a mild elevation of BAL eotaxin levels has been demonstrated in asthmatics compared to CEP (median; 53 $pg \cdot mL^{-1}$) using the same eotaxin assay system as utilized in this study [4]. There were no significant differences in the chemokines and IL-5 concentrations in the BAL fluid between CEP patients who had experienced asthmatic symptoms and those who had not in the present study (data not shown). Th2 dominant lymphocyte activation is a characteristic of asthmatics, while both Th1 and Th2 cells may be involved in CEP [16]. Precise mechanisms accounting for the enhanced eosinophil-related reaction with CEP are unknown, but the present study suggests that markedly higher eotaxin levels are involved in augmented eosinophilic inflammation. The presence of undetected allergen exposure or genetic differences cannot be excluded in the pathogenesis of CEP. In addition, it should be noted that there were two CEP patients with BAL eotaxin levels that were nearly undetectable despite the presence of significant eosinophil accumulation (table 1, fig. 2a) and that other factors are capable of recruiting eosinophils into the lung. These observations indicate that CEP may not be a single disorder in terms of

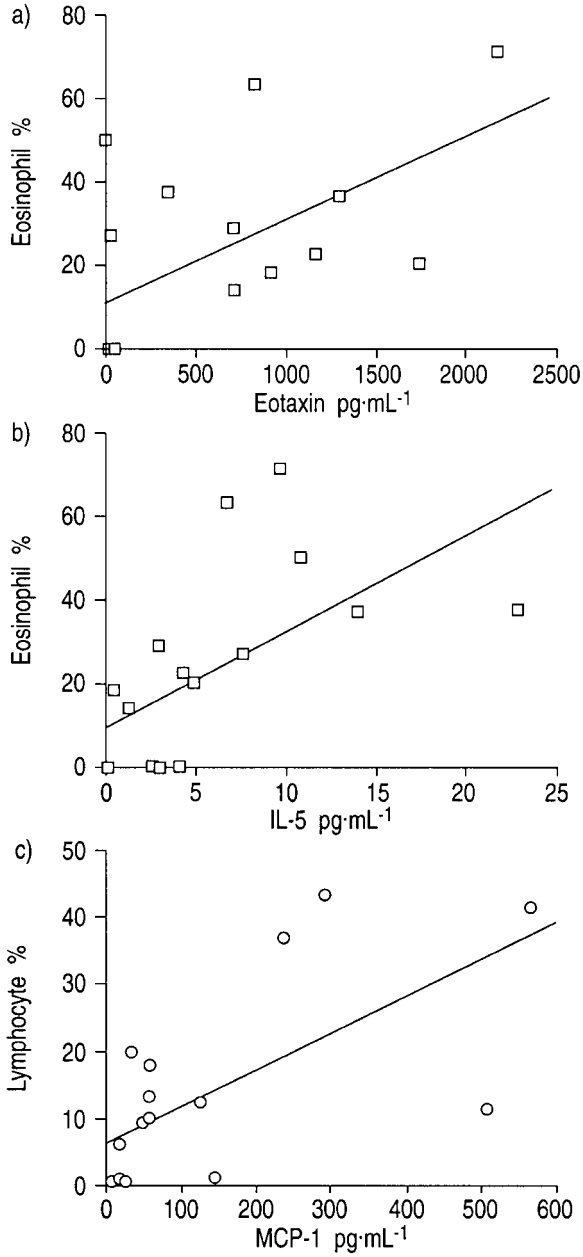

Fig. 3. – Simple linear regression analysis between the mediator concentrations and the proportions of eosinophils or lymphocytes in bronchoalveolar fluid. Data for chronic eosinophilic pneumonia (CEP) (n = 11) and control (CTL) (n = 6) subjects has been included in each figure. Correlations between a) %eosinophil and eotaxin (r = 0.582, p = 0.014); b) interleukin (IL)-5 levels (r = 0.575, p = 0.016); c) between %lymphocyte and monocyte chemoattractant protein (MCP)-1 levels (r = 0.662, p = 0.004) are presented.

eotaxin elaboration. The authors, however, found no clinical correlate of low eotaxin CEP in this study (table 1).

A previous report suggested that eotaxin production and eosinophil recruitment to the airway were diminished by the depletion of T-lymphocytes in a mouse model of allergic inflammation [17]. Both Th1 and Th2 cytokines enhanced cytokine-induced eotaxin expression in airway epithelial cells [4, 18]. In addition, activated helper T-cells had increased in number [19]

Table 2. – Multiple linear regression analysis between concentrations of the mediators and proportions of eosinophils and lymphocytes in bronchoalveolar lavage fluid

	% Eosinophils			% Lymphocytes		
	SPRC	t-values	p-values	SPRC	t-values	p-values
Eotaxin	0.460	2.52	0.028	0.239	1.31	0.134
MCP-1	0.272	1.27	0.230	1.060	4.96	0.0004
IL-5	0.741	2.43	0.033	-0.136	-0.45	0.664
RANTES	0.197	1.15	0.273	0.028	0.17	0.872
MIP-1α	-0.458	-1.53	0.154	-0.491	-1.64	0.129

SPRC: standard partial regression coefficient; MCP-1: monocyte chemoattractant protein-1; IL-5: interleukin-5; RANTES: regulated on activation, normal T-cell expressed and secreted; MIP-1α: macrophage inflammatory protern-1α.

and both Th1 and Th2 cytokines have been observed in the airways of CEP patients [16]. These observations are consistent with the current findings, *i.e.* marked elevation of eotaxin levels is accompanied by lymphocyte accumulation and the elevation of IL-5 and MCP-1, both of which are potentially associated with T-lymphocyte activation. It is also possible that increased eotaxin in the airways may directly affect lymphocytes as CCR3 has been reported to be expressed on some Th2 cells [20]. No significant correlation was observed between eotaxin levels and the relative lymphocyte numbers in BAL fluid (table 2), but the contribution of eotaxin to recruitment and activation of Th2 lymphocytes in CEP cannot be excluded since subpopulations of lymphocytes were not discriminated in the present study. Although the main physiological role of MCP-1 is thought to be the activation of monocytic cells [21, 22], MCP-1 is also active on activated T-cells which express CCR2. The present findings suggest that MCP-1 contributes to lymphocyte accumulation in CEP (table 2 and fig. 3). RANTES can act on lymphocytes as well since it can bind to CCR1 and CCR5 in addition to CCR3 [21]. The current results indicate the presence of slightly elevated RANTES levels in CEP that seems to be consistent with previous research [13] (fig. 2b). Although the present study could not find significant correlations between RANTES or MIP-1α levels and percentages of BAL eosinophils, it does not immediately mean lack of implication of these CC chemokines on eosinophils influx, since other factors not explored in this study may be implicated in the chemotaxis, for instance, the expression levels of CCR3 on eosinophils, the interaction between very late activation antigen-4 on eosinophils and vascular cell adhesion molecule-1 on endothelial cells. Furthermore, CC chemokines and IL-5 may act in a coordinated fashion [9].

It is interesting that the proportion of lymphocytes was higher in patients with sarcoidosis, but the MCP-1 levels were lower than those in CEP patients (figs. 1 and 2). These observations are attributed to the fact that Th1 dominant lymphocyte activation occurs without the aid of MCP-1 in sarcoidosis [23], while lymphocyte activation observed in CEP is similar to allergic inflammation including asthma in that the Th2 cytokine IL-5 and MCP-1 levels have increased [12]. In contrast, MCP-1 levels were higher in pulmonary

fibrosis, but the lymphocyte counts were lower than those in CEP patients. It is to be elucidated why marked lymphocyte accumulation is not induced in the presence of abundant MCP-1 in pulmonary fibrosis. The differential status of activated alveolar macrophages may be responsible for the distinct differentials of BAL cells in CEP, sarcoidosis, and pulmonary fibrosis.

Although IL-5 levels were higher in CEP patients than those in the CTL group and significantly correlated with the percentage of eosinophils (figs. 2 and 3, table 2), the levels in BAL fluid were relatively low when compared to those previously reported in patients with eosinophilic pneumonia [24, 25]. Previous reports described high IL-5 levels in BAL fluid, mainly in acute eosinophilic pneumonia. The authors also found a patient with acute eosinophilic pneumonia whose BAL IL-5 level was >1 ng·mL^{-1} (data not shown) applying the identical kit used for this study, indicating that the pathogenesis of acute eosinophilic pneumonia differs considerably from that of CEP in that a more remarkable but transient release of IL-5 may occur in acute eosinophilic pneumonia. In addition, IL-5 has been reported to be released from eosinophils *per se* besides Th2 lymphocytes [26]. These observations indicate a positive regulation of eosinophil through autocrine secretion of IL-5 in the airways of CEP patients.

In conclusion, a significant correlation between bronchoalveolar lavage eotaxin levels and eosinophils, and bronchoalveolar lavage monocyte chemoattractant protein-1 levels and lymphocytes in patients with chronic eosinophilic pneumonia has been demonstrated, suggesting that these chemokines contribute to the recruitment of inflammatory cells in the lung in chronic eosinophilic pneumonia.

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