

## Elevated chemokine levels in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia

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**Background:** Allergic lung inflammation is caused by accumulation and activation of different leukocyte subsets, such as eosinophils and T lymphocytes, in the lung. The chemokines are a large group of chemotactic cytokines that regulate leukocyte trafficking and may play an important role in allergic lung inflammation.

**Objective:** The purpose of this study was to evaluate the role of various chemokines, including eotaxin, RANTES, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\beta$ , and IL-8 in the pathogenesis of eosinophilic pneumonia (EP).

**Methods:** The concentrations of eotaxin, RANTES, MCP-1, MIP-1 $\beta$ , and IL-8 in bronchoalveolar lavage fluid (BALF) were measured by using ELISA in 15 patients with EP, 10 with idiopathic pulmonary fibrosis, 10 with sarcoidosis, and 11 healthy volunteers.

**Results:** Eotaxin in BALF was high only in patients with EP, and its level correlated significantly with the number of eosinophils in BALF of patients with EP and healthy volunteers. MCP-1 and MIP-1 $\beta$  in BALF were preferentially increased in patients with EP. There was a significant correlation between MCP-1 levels and the number of macrophages in BALF of patients with EP and healthy volunteers.

**Conclusion:** Our findings suggest that these CC chemokines contribute to the pathogenesis of EP through the specific recruitment of leukocyte subsets in the lung. (*J Allergy Clin Immunol* 2000;106:730-6.)

**Key words:** Eosinophilic pneumonia; eotaxin; RANTES; monocyte chemoattractant protein 1; macrophage inflammatory protein 1 $\beta$ ; IL-8; bronchoalveolar lavage fluid; eosinophil

Eosinophilic pneumonia (EP) is an inflammatory disease of the lung characterized by increased influx of eosinophils.<sup>1-3</sup> However, the underlying mechanism of

### Abbreviations used

BALF:	Bronchoalveolar lavage fluid
EP:	Eosinophilic pneumonia
IPF:	Idiopathic pulmonary fibrosis
MCP:	Monocyte chemoattractant protein
MIP:	Macrophage inflammatory protein

eosinophil accumulation into the lung of patients with EP is still unclear. Previous studies have indicated that the production of IL-5 might be involved in the pathogenesis of EP.<sup>4,5</sup> We have also recently reported that local production of IL-5 might be important in patients with EP.<sup>6</sup>

Eotaxin is a newly discovered chemokine that has selective chemotactic activity for eosinophils.<sup>7</sup> Intratracheal administration of eotaxin in mice induces a rapid and substantial accumulation of eosinophils in bronchoalveolar lavage fluid (BALF).<sup>8</sup> It has been suggested that IL-5 is essential for priming and increasing the pool of available eosinophils, whereas eotaxin is required to attract these cells into the airways of various animal models.<sup>9-11</sup> Targeted disruption of the eotaxin gene partially reduced antigen-induced tissue eosinophilia in vivo.<sup>12</sup> Eotaxin may thus be important in EP by recruiting eosinophils into the airways.

The recruitment and activation of other leukocytes, such as T cells,<sup>13</sup> has also been described in patients with EP. Chemokines are initially divided into two major subfamilies, CXC and CC. The former, typified by IL-8, are mostly active on neutrophils, whereas the latter chemokines, such as RANTES, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1 $\beta$ , have preferential activity on mononuclear leukocytes.<sup>14</sup> Interestingly, RANTES selectively attracts memory subtype CD45RO<sup>+</sup>CD4<sup>+</sup> T cells and is also chemotactic for eosinophils.<sup>15,16</sup> On the other hand, MCP-1 is primarily involved in the recruitment of mononuclear phagocytes,<sup>17</sup> and MIP-1 $\beta$  is predominantly chemotactic for CD4<sup>+</sup> T cells.<sup>18</sup> Furthermore, it has been demonstrated that RANTES and MIP-1 $\beta$  induce the binding of CD4<sup>+</sup> T cells to the extracellular matrix.<sup>19</sup>

We hypothesized that the selective recruitment of leukocytes to the lung in patients with EP might reflect

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the local production of chemokines. In this study we investigated the production of eotaxin, RANTES, MCP-1, MIP-1 $\beta$ , and IL-8 in the lung. Chemokine protein levels in cell-free BALF were measured by using ELISA. To characterize the immunologic mechanism of EP, results were compared with healthy volunteers, patients with idiopathic pulmonary fibrosis (IPF), and patients with sarcoidosis. Furthermore, we also examined the relationship between chemokine levels and leukocyte counts in BALF.

## METHODS

### Patient population

Included in this study were 15 untreated patients with EP (9 women and 6 men; age,  $42.8 \pm 16.8$  years), 10 with IPF (3 women and 7 men; age,  $62.8 \pm 6.3$  years), 10 with sarcoidosis (9 women and 1 man; age,  $47.9 \pm 13.9$  years), and 11 healthy adults (3 women and 8 men; age,  $24.2 \pm 4.4$  years). None of the patients in this study was treated with corticosteroid at the time of the investigation. Seven patients with EP, 5 with IPF, 1 with sarcoidosis, and none of the volunteers were smokers. None of the patients had atopic status, except two patients with EP. Statistically, both smoking and atopic status had no influence on the BALF chemokine levels (data not shown).

The diagnosis of EP was based on clinical criteria: patients with no known cause of EP (drugs, parasitic infection, and fungal disease), with acute or chronic dyspnea, interstitial infiltrates on chest radiographs, pulmonary eosinophilia, prompt resolution of clinical and radiographic abnormalities with or without corticosteroid therapy, and histologic evidence based on examination of transbronchial lung biopsy specimens. The diagnosis of IPF was based on the following criteria: negative history of exposure to environmental agents known to cause interstitial lung disease, negative history of chronic lung infections, evidence of interstitial infiltrates on chest roentgenography and chest computed tomography scanning, restrictive ventilatory defect pattern on pulmonary function tests, histologic evidence of interstitial pneumonitis with varying degrees of interstitial fibrosis without granulomas, and histologic features of usual interstitial pneumonia confirmed by surgical lung biopsy. The diagnosis of sarcoidosis was based on examination of biopsy specimens obtained from lungs, lymph nodes, or skin showing non-caseating epithelioid cell granulomas, with no evidence of inorganic material known to cause granulomatous diseases.

### Bronchoalveolar lavage

After obtaining informed consent from the subject, bronchoalveolar lavage was performed by using a flexible fiberoptic bronchoscope under local anesthesia of the upper airway with 2% lidocaine, as described previously.<sup>20</sup> Briefly, the bronchoscope was wedged into the subsegmental bronchus of the right middle lobe or, in patients with EP, into areas of lung parenchyma otherwise normal on chest roentgenography, and 150 mL of normal saline was instilled in 50-mL aliquots. Harvested BALF was filtered through sterile nylon mesh and centrifuged at 160g for 10 minutes to obtain the cell preparation. The cells were later stained by the May-Giemsa method, and a differential count was performed on 200 cells. The remaining fluid was centrifuged at 500g for 5 minutes, and the supernatant was stored at  $-80^{\circ}\text{C}$  until use. There were no differences in storage time between groups. Furthermore, the correlations between storage time and chemokine concentrations were not found (data not shown).

### Measurement of eotaxin, RANTES, MCP-1, MIP-1 $\beta$ , and IL-8 concentrations in BALF

BALF concentrations of eotaxin, RANTES, MCP-1, and MIP-1 $\beta$  were measured by using the respective ELISA kits (R&D systems).

IL-8 concentration in BALF was measured by using an ELISA kit (CLB). The detection limits were 5.0, 5.0, 5.0, 5.0, and 2.0 pg/mL for eotaxin, RANTES, MCP-1, MIP-1 $\beta$ , and IL-8, respectively. The upper limits of the assays were 1000, 2000, 1000, 1000, and 600 pg/mL for eotaxin, RANTES, MCP-1, MIP-1 $\beta$ , and IL-8, respectively. BALF was diluted with saline in some cases to get on the standard curve. Concentrations below the lower limit of detection were assumed to be zero for the purpose of statistical analysis. The absolute levels of cytokines in BALF rather than those relative to proteins were reported in this study because cytokine levels in BALF are not influenced by the concentration of albumin, as demonstrated in our previous study.<sup>20</sup> The mean fluid recoveries were detailed in Table I. There were no differences of the percentage recovery of lavage fluid among the subject groups. Furthermore, there was no correlation between the percentage recovery of lavage fluid and the chemokine levels in patients with EP and healthy volunteers (not shown).

### Statistical analysis

All data were expressed as mean  $\pm$  SEM. Comparisons of paired data were carried out by using Kruskal-Wallis ANOVA followed by the Scheffé F test. Differences with *P* values of less than .05 were considered significant. Correlations between two variables were examined by using the Pearson correlation coefficient. Statistical comparisons of the total number and differential count of leukocytes in BALF were performed by using the Mann-Whitney *U* test (Table I).

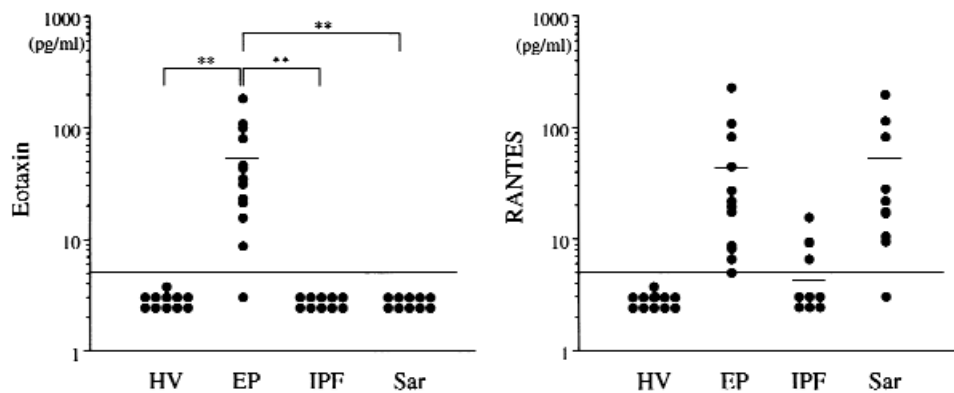
## RESULTS

### Characteristics of BALF cells

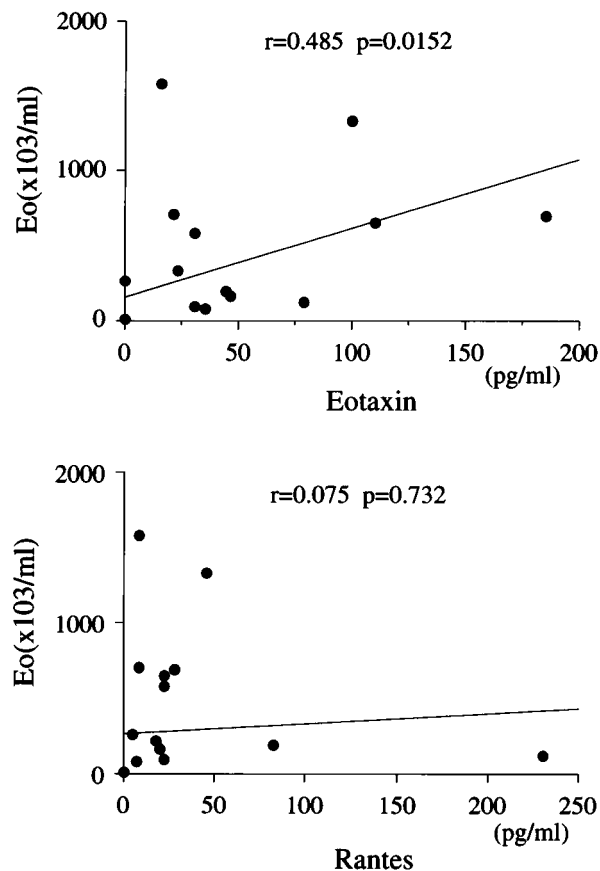
The total number of leukocytes and differential counts in BALF are summarized in Table I. The total number of cells per milliliter of BALF in each patient group was higher than that in healthy subjects. Differential cell counts showed that the percentage of eosinophils in EP and IPF, but not in sarcoidosis, was significantly higher than the control values (*P* < .01 and *P* < .05, respectively; Table I). Furthermore, the absolute number of eosinophils was higher in patients with EP compared with patients with IPF and sarcoidosis and healthy volunteers (*P* < .05, not shown). The percentage of lymphocytes was significantly higher in patients with sarcoidosis (*P* < .05) than in healthy subjects (Table I). In addition to the increased number of eosinophils, the absolute number of lymphocytes was higher in patients with EP compared with healthy volunteers (*P* < .05, not shown). The percentage of neutrophils in patients with EP and IPF was significantly higher than in healthy subjects (*P* < .05 and *P* < .01, respectively; Table I). The absolute number of neutrophils was also higher in patients with EP compared with healthy volunteers and patients with sarcoidosis (*P* < .05, not shown).

### Eotaxin and RANTES concentrations in BALF

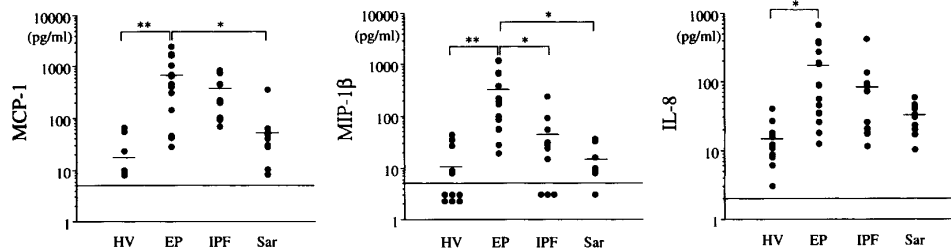
To investigate the possible role of chemokines in various interstitial lung diseases, two typical CC chemokines chemotactic for eosinophils (eotaxin and RANTES) were assessed in BALF. Interestingly, eotaxin was detected only in patients with EP ( $52.1 \pm 13.5$  pg/mL, Fig 1). The levels of RANTES in BALF were higher in all disease groups compared with healthy volunteers (EP:  $44.6 \pm 16.4$  pg/mL; IPF:  $4.1 \pm 1.8$  pg/mL; sarcoidosis:  $50.1 \pm$



**FIG 1.** Concentrations of eotaxin and RANTES in BALF of patients with EP, IPF, sarcoidosis (*Sar*), and healthy volunteers (*HV*). Short horizontal lines represent the mean values. Horizontal lines represent the detection limit of each chemokine. \*\* $P < .01$  compared with each of the other groups.



**FIG 2.** Relationship between eosinophilia and eotaxin and RANTES levels in BALF. Correlations between the absolute number of eosinophils (*Eo*) and concentration of eotaxin in BALF (*top*, patients with EP and healthy volunteers) and the concentration of RANTES in BALF (*bottom*, patients with EP and healthy volunteers) are shown.



**FIG 3.** Concentrations of MCP-1, MIP-1β, and IL-8 in BALF of different diagnostic groups. *Short horizontal lines* represent the mean values. *Horizontal lines* represent the detection limits of each chemokine. \**P* < .05 compared with each of the other groups; \*\**P* < .01 compared with each of the other groups. *Sar*, Sarcoidosis; *HV*, healthy volunteers.

**TABLE I.** Characteristics of BALF cells

	Recovery (%)	Total cells (10 <sup>5</sup> /mL)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
Healthy volunteers (n = 11)	61.0 ± 3.2	1.4 ± 0.2	85.1 ± 2.6	11.9 ± 2.5	2.0 ± 1.2	1.0 ± 0.7
EP (n = 15)	56.3 ± 3.7	11.3 ± 2.5 <sup>†</sup>	24.0 ± 4.0 <sup>†</sup>	17.7 ± 3.2	7.7 ± 2.7*	50.1 ± 5.9 <sup>†</sup>
IPF (n = 10)	52.8 ± 4.1	4.4 ± 0.6 <sup>†</sup>	68.9 ± 6.3*	18.8 ± 5.7	6.6 ± 2.3 <sup>†</sup>	5.6 ± 2.8*
Sarcoidosis (n = 10)	52.4 ± 4.1	2.5 ± 0.4*	61.6 ± 8.1*	37.3 ± 8.2*	1.0 ± 0.5	0.1 ± 0.1

\**P* < .05 compared with healthy volunteer subjects.

<sup>†</sup>*P* < .01 compared with healthy volunteer subjects.

20.2 pg/mL), but the difference between each group was not significant (Fig 1). Furthermore, there was a significant correlation between eotaxin concentrations and absolute eosinophil numbers ( $r = 0.485$ ,  $P < .0152$ ; Fig 2) in BALF of patients with EP and healthy volunteers. However, there was no relationship between eosinophil count and RANTES concentrations in BALF of patients with EP, even if healthy volunteers were included (Fig 2).

### MCP-1, MIP-1β, and IL-8 concentrations in BALF

Other chemokines may be involved in selective leukocyte recruitment in patients with EP. In the next step we measured the level of MCP-1 and MIP-1β, chemotactic factors for mononuclear cells, and IL-8, a chemotactic factor for neutrophils, in BALF. Furthermore, we also determined the relationships between these chemokines and the number of leukocytes. The mean concentration of MCP-1 in BALF of patients with EP ( $743.5 \pm 214.8$  pg/mL) was significantly elevated compared with patients with sarcoidosis ( $69.1 \pm 32.8$  pg/mL,  $P < .05$ ) and healthy subjects ( $19.6 \pm 6.1$  pg/mL,  $P < .01$ ; Fig 3). MIP-1β levels were also higher in patients with EP ( $357.6 \pm 109.9$  pg/mL) compared with those with sarcoidosis ( $17.0 \pm 4.0$  pg/mL,  $P < .05$ ), IPF ( $48.3 \pm 23.1$  pg/mL,  $P < .05$ ), and healthy subjects ( $11.0 \pm 4.8$  pg/mL,  $P < .01$ ; Fig 3). Furthermore, IL-8 level was higher in BALF of patients with

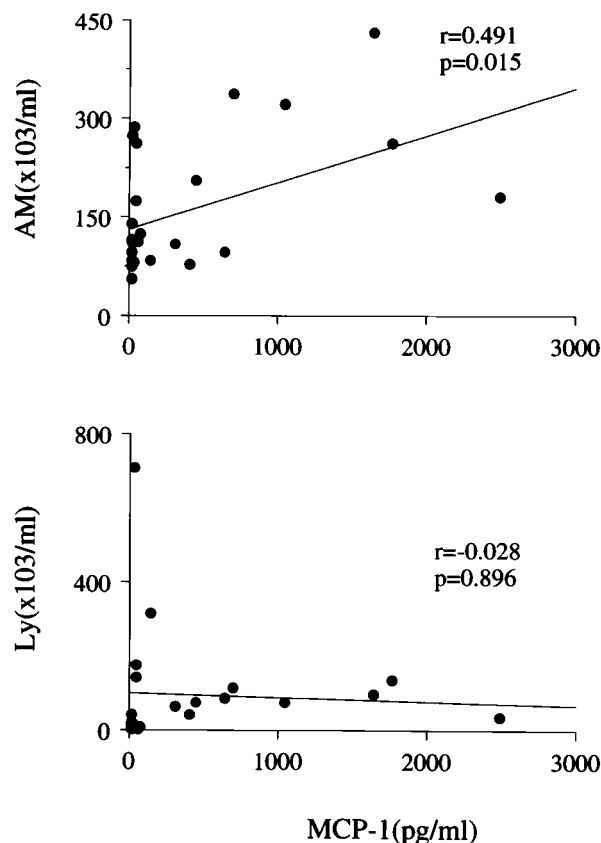
EP ( $173.2 \pm 48.5$  pg/mL) compared with that of healthy volunteers ( $15.5 \pm 3.0$  pg/mL,  $P < .05$ ; Fig 3).

There was a significant correlation between MCP-1 levels and macrophage count ( $r = 0.491$ ,  $P = .015$ ) but not lymphocyte counts in BALF of patients with EP and healthy volunteers (Fig 4). The level of MIP-1β in BALF did not correlate with the number of mononuclear cells (macrophages and lymphocytes) in BALF (data not shown). There was a significant correlation between IL-8 levels and the number of neutrophils in patients with EP and healthy volunteers ( $r = 0.667$ ,  $P = .0002$ ; data not shown).

### DISCUSSION

EP is characterized by the accumulation in the lung of not only eosinophils but also activated T lymphocytes.<sup>4</sup> Furthermore, lymphocytes recruited and activated by antigen-presenting cells release cytokines locally in the lung, such as IL-5, which regulate eosinophil recruitment and activation.<sup>6,21</sup> Previous studies have also shown that B7 costimulatory molecules on antigen-presenting cells could also play an important role in pulmonary eosinophilia.<sup>22</sup> The selective accumulation of these inflammatory cells into the lung is thought to be critical in the pathogenesis of EP.

Eotaxin, a potent and eosinophil-specific chemoattractant, selectively induces eosinophil accumulation in



**FIG 4.** Relationship between mononuclear cells and MCP-1 levels in BALF. Correlations between the concentration of MCP-1 and the absolute number of alveolar macrophages (AM) in BALF (*top*, patients with EP and healthy volunteers) and the absolute number of lymphocytes (Ly) in BALF (*bottom*, patients with EP and healthy volunteers) are shown.

allergic animal models.<sup>8,23</sup> It has been suggested that overexpression of eotaxin in BALF and airways contributes to the chemotaxis of eosinophils to the site of inflammation in human subjects.<sup>24</sup> Although RANTES has been reported as a selective chemotactic factor for memory T cells,<sup>15</sup> this chemokine is also chemotactic for eosinophils.<sup>16</sup> In the present study we investigated the mechanism of eosinophil infiltration into the lung in patients with EP by examining the concentrations of these eosinophil chemotactic factors in BALF and compared their levels with those found in patients with IPF and sarcoidosis. To our knowledge, our study is the first to demonstrate increased BALF concentrations of eotaxin in EP among various interstitial lung diseases (Fig 1). We also identified a significant correlation between eotaxin level and the number of eosinophils in BALF of patients with EP and healthy volunteers (Fig 2). Although the levels of RANTES in BALF were elevated in both EP and sarcoidosis (Fig 1), the number of eosinophils did not correlate with the concentration of

RANTES in BALF of patients with EP, even if healthy volunteers were included (Fig 2). Interestingly, there was a significant correlation between the number of lymphocytes and RANTES concentration in BALF of patients with sarcoidosis and healthy subjects ( $r = 0.72$ ,  $P < .0001$ ; data not shown). These findings suggest that the role of eotaxin in the accumulation of eosinophils in the lung of patients with EP is more critical than that of RANTES. Studies from our laboratory have also shown elevated concentrations of IL-5 in the lung and peripheral blood of patients with EP.<sup>6</sup> Furthermore, the cooperation between IL-5 and eotaxin to induce eosinophil accumulation has been reported by several investigators.<sup>9-11</sup> Taken together, these results indicate that not only IL-5 but also eotaxin could play an important role in lung eosinophilia in patients with EP.

MCP-1 is chemotactic for both monocytes and memory T lymphocytes.<sup>25-27</sup> Furthermore, the contribution of MCP-1 to type 2 cytokine-mediated inflammation is more significant than that of type 1 cytokines.<sup>28</sup> Interest-

ingly, elevated levels of MCP-1 in BALF of patients with EP were identified in our study compared with those of healthy subjects and sarcoidosis (Fig 3). Furthermore, the number of macrophages, but not lymphocytes, correlated with MCP-1 levels in BALF of patients with EP if healthy volunteers were included (Fig 4). These results suggest that MCP-1 could be chemotactic for monocytes in the lung of patients with EP. It has been recently reported that MCP-1 neutralization diminishes allergic inflammation of the lung.<sup>29</sup> Previous reports and our data demonstrated that MCP-1 could be one of the important factors in eosinophilic lung diseases.

MIP-1 $\beta$  exhibits a preferential chemotactic effect on CD4<sup>+</sup> T cells.<sup>18</sup> In our study elevated levels of MIP-1 $\beta$  were found in BALF of patients with EP compared with that found in other groups. However, there was no correlation between concentrations of MIP-1 $\beta$  and the number of CD4<sup>+</sup> T cells in BALF of patients with EP, even if healthy volunteers were included, but the number of eosinophils correlated with levels of MIP-1 $\beta$  in BALF of patients with EP and healthy volunteers ( $r = 0.489$ ,  $P = .0122$ ; data not shown). These results suggest that MIP-1 $\beta$  may play some role in lung eosinophilia of patients with EP by attracting a subpopulation of T cells.

IL-8 is considered as the most potent chemotactic factor for polymorphonuclear leukocytes.<sup>14</sup> In our study we found a significant correlation between IL-8 levels and the number of neutrophils but not that of eosinophils in BALF of patients with EP and healthy volunteers (data not shown). These findings suggest that IL-8 is more likely to be involved in the infiltration of neutrophils into the lung in patients with EP.

In conclusion, we have demonstrated in the present study that the levels of CC chemokines, eotaxin, MCP-1, and MIP-1 $\beta$  were preferentially elevated in BALF of patients with EP among several interstitial lung diseases. These CC chemokines could play an important role in eosinophilic inflammation of the lung in patients with EP. Further studies, including the expression of chemokine receptors on inflammatory cells, are necessary to clarify the role of CC chemokines in eosinophilic lung diseases.

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