

## Interleukin 5 Levels in Bronchoalveolar Lavage Fluid from Patients with Interstitial Lung Disease

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Bronchiolitis obliterans organizing pneumonia (BOOP) is clinically and histologically difficult to differentiate from chronic eosinophilic pneumonia (CEP). The common histological feature of these diseases is an infiltration of mononuclear cells into the small airway, interstitial tissue and alveoli. Activation of T cells and secretion of lymphokines play a crucial role in the regulation and coordination of immune responses and inflammatory processes. In this study, we demonstrated the distribution of lymphocyte subpopulations and their stage of activation and T cell-derived cytokine, IL-5 level in bronchoalveolar lavage (BAL) fluid obtained from patients with BOOP and CEP by comparison with sarcoidosis and diffuse panbronchiolitis (DPB). Flow cytometric analysis of T cell activation markers revealed that BOOP and sarcoidosis are characterized by increased absolute numbers of HLA-DR-bearing T cell subsets, while the relative numbers were increased in CEP and DPB. IL-5 in BAL fluids from BOOP and CEP was significantly high levels, and CEP patients with the high level of IL-5 showed the marked elevation of BAL eosinophils. Furthermore, it is of interest that the absolute numbers of HLA-DR T cells correlated with IL-5 levels in BAL fluid. These results suggest that activated T cells and secretion of IL-5 may be important factors in the pathogenetic processes of BOOP and CEP.

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**Key Words :** BOOP, Chronic eosinophilic pneumonia, IL-5, Activated T cells

### Introduction

Interleukin 5 (IL-5) is an important inflammatory cytokine which represents an eosinophil proliferation and differentiation factor and is chemotactic for eosinophils<sup>1-3)</sup>. Recently, activated T cells and T cell-derived cytokine was found to be related to the eosinophilia found in both allergic and nonallergic asthmatic individuals, and IL-5 can prolong eosinophil survival *in vitro*<sup>4)</sup>. This cytokine has been reported to be produced from helper T (Th) cells, especially from Th 2 cells which are involved in immunoglobulin production and allergic reaction<sup>5)</sup>.

Chronic eosinophilic pneumonia (CEP) is characterized histologically by the eosinophilic exudation into the

interstitial tissue and small air spaces mixed with mononuclear and multinucleated cells<sup>6,7)</sup>. Because CEP shows symptoms, chest roentgenographic findings and corticosteroid response similar to those of bronchiolitis obliterans organizing pneumonia (BOOP) proposed by Epler et al<sup>8)</sup>, which is defined pathologically by polypoid masses of granulation tissues in the lumen of small airways, alveolar ducts and some alveoli, and a variable interstitial infiltration of mononuclear cells<sup>6,8)</sup>, CEP is considered as the most important disease to be differentiated from BOOP. These observations lead us to the possibility that T cells may release IL-5 and this cytokine may be associated with eosinophilia in CEP.

It is likely that tissue lymphocytes contribute more to eosinophilia and/or IL-5 production than blood lymphocytes. However, according to our knowledge, no reports have been published regarding to the relationship between IL-5 level and a variety of cells infiltrating into the inflammatory site of the lung. Thus, the aim of this study was to analyze the distribution of lymphocyte subpopulations and their stage of activation and T cell-derived cytokine, IL-5 level in bronchoalveolar lavage (BAL) fluid obtained from patients with CEP and BOOP. Sarcoidosis and diffuse panbronchiolitis (DPB), which is characterized by lymphocytosis<sup>9)</sup> and neutrophilia<sup>10)</sup> in BAL fluid, were also investigated in the same way as a disease control.

### Methods

#### Subjects

We studied 8 patients with BOOP (2 women and 2 men, aged 54 to 81 yr) and CEP (2 women and 2 men, aged 48 to 74 yr), 9 patients with sarcoidosis (all women, aged 27 to 72 yr), 10 patients with DPB (3 women and 7 men, aged 22 to 73 yr) and 5 healthy volunteers (1 woman and 4 men, aged 20 to 39 yr). All BOOP patients were diagnosed by clinicopathological evaluation (1 by open lung biopsy and 3 by transbronchial lung biopsy). Chest X ray showed alveolar opacities in 3 cases and interstitial opacities in the other. There was no evidence of an underlying

disorder or known cause that is associated with BOOP. The diagnosis of CEP was made according to classical clinical criteria<sup>7)</sup>. The patients had typical clinical features consistent with CEP, together with chest X-rays and pathological evidence of eosinophil infiltration in the alveolar walls and spaces by transbronchial lung biopsy. All CEP patients showed peripheral eosinophilia ( $26.3 \pm 22.9\%$ ) and the typical progressive peripheral dense infiltrates on chest X-ray with unknown etiology. Sarcoidosis was diagnosed by examination of biopsy specimens obtained from lungs, lymph nodes, or skin showing noncaseating epithelioid cell granulomas, with no evidence of inorganic material known to cause granulomatous disease. All patients had clinically active disease with new pulmonary or general symptoms and signs, chest radiographic abnormalities, and a positive <sup>67</sup>Gallium scan. Eight patients with DPB were diagnosed clinically by meeting the clinical diagnostic criteria published by the Japanese Ministry of Health and Welfare, and the other 2 patients were diagnosed pathologically by open lung biopsy. All 10 patients had persistent cough and sputum for more than 2 yr and exertional dyspnea. Chest roentgenogram demonstrated diffuse fine nodular shadows in both lungs in all patients and overinflation in 6 patients. When patients had signs or roentgenographic findings suggesting pneumonia or acute exacerbation of the disease before enrollment in the study, adequate antibiotics were administered. Thus, none had a pulmonary infection in the 1 month before enrollment in the study. At the time of the investigation, none were treated with corticosteroids or antibiotics.

#### *Bronchoalveolar Lavage and Cell Preparations*

Under the informed consent of patients, BAL was performed by a flexible fiberoptic bronchoscope (Olympus BF-P20 type, Olympus Corp., Tokyo) after local anesthesia of the upper airway with 4% lidocaine. The tip of the fibroscope was advanced to a wedged position into the subsegmental bronchus of the right middle lobe or into areas of lung parenchyma otherwise normal on chest X-ray in patients with peripheral opacities, and three or four 50-ml aliquots were sequentially instilled followed by immediate aspiration after each aliquot. The BAL fluid was filtered through sterile nylon mesh to remove large particles of mucus and then centrifuged (Cytospin 2, Shandon Instruments, Sewickley, PA) at 1,100 rpm for 2 min to obtain the cell preparation. The cell pellets were stained with May-Giemsa method, and a differential count was performed on 200 cells. The remaining fluid was centrifuged at 500 xg for 5 min, and the supernatant was stored at -80 °C until studied for the cytokine assay. The cell pellets were resuspended in PRMI-1640 medium (Gibco, Paisley Scotland) supplemented with 10% fetal calf serum, and incubated in plastic dishes for 60 min at

37 °C in humidified 5% CO<sub>2</sub>-air. More than 90% of nonadherent cells collected for flow cytometric analysis were viable by the trypan blue exclusion test.

#### *Two-Color Flow Cytometry*

The BAL fluid cells were adjusted to  $1 \times 10^7$  cells per milliliter. A total of 10  $\mu$ l of each monoclonal antibody was placed into a polystyrene tube, and 50  $\mu$ l of the cell suspension ( $5 \times 10^5$  cells) was added. Cells were incubated for 30 min on ice in the dark, washed once in cold phosphate-buffered saline solution (PBS) containing 0.1% sodium azide, and then resuspended in cold PBS containing 0.5% paraformaldehyde. The fixed cells were kept in the dark at 4 °C. Stained cells were analyzed on a flow cytometer (FACScan, Becton Dickinson, FACS Division). A computer system (Consort 30, Becton Dickinson) was used for data acquisition and analysis. List mode data for 10,000 to 20,000 events were stored. A cell gate containing lymphocytes was established on the basis of forward and side light scatter. To determine the border line between stained and unstained cells, cells were also stained with mouse IgG1 and IgG2a conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Percentages were calculated based on the number of lymphocytes found in each quadrant. Interassay reproducibility was checked using beads (CALIBRITE, Becton Dickinson) and software (AutoCOMP, Becton Dickinson).

#### *Monoclonal antibodies*

FITC-conjugated anti-HLA-DR, CD25 (IL-2R $\alpha$ ) and CD8 (Leu-2) antibodies, and PE-conjugated anti-CD3 (Leu-4), CD4 (Leu-3) antibodies were purchased (Becton Dickinson, Mountain View, CA). Mouse IgG1 and IgG2a conjugated with FITC or PE were purchased (Coulter Immunology) and used to determine the borderline between stained and unstained cells in a flow cytometric analysis.

#### *Measurement of interleukin 5*

The level of IL-5 was quantified using enzyme-linked immunosorbent assay kit (kindly provided from Suntory Ltd, Osaka). Briefly, 100  $\mu$ l of monoclonal antibody to human IL-5 (10  $\mu$ g/ml) was bound to microtiter plates by incubating at 4 °C overnight. The wells were washed three times with PBS containing 0.1% Tween 20, pH 7.2 (buffer A). After blocking with 200  $\mu$ l of 1% bovine serum albumin in PBS, 100  $\mu$ l of BAL supernatant and recombinant human IL-5 for standard were added to each well and incubated at room temperature overnight followed by washing 5 times with buffer A. 100  $\mu$ l of second antibody (polyclonal antibody to human IL-5) was added to each well and incubated at room temperature for 4 h, and then

**Table 1** Eosinophils and lymphocyte subpopulations in bronchoalveolar lavage fluid from patients with interstitial lung diseases compared to healthy volunteers

| Cells*        | Healthy Volunteers (A) | BOOP (B) | CEP (C)  | Sarcoidosis (D) | DPB (E) | significant differences among the groups |
|---------------|------------------------|----------|----------|-----------------|---------|--|
| Eosinophils   | 0.1±0.1                | 1.2±0.2  | 14.3±3.2 | 0.1±0.0         | 0.6±0.2 | C/A, B, D, E*                            |
| Lymphocytes   | 1.0±0.1                | 12.7±4.3 | 5.0±2.1  | 12.3±3.6        | 5.7±2.0 | B, D/A**                                 |
| CD3           | 0.7±0.1                | 10.6±3.4 | 4.4±1.9  | 11.0±3.4        | 5.1±1.9 | B, D/A**                                 |
| CD4           | 0.3±0.1                | 4.0±1.6  | 2.6±1.1  | 8.8±2.9         | 2.3±1.2 | D/A*, E**                                |
| CD8           | 0.3±0.1                | 6.9±2.5  | 1.5±0.7  | 2.3±0.7         | 2.4±0.7 | B/A†, C, D, E†                           |
| CD4/CD8 ratio | 1.2±0.2                | 0.7±0.4  | 1.8±0.5  | 4.4±0.8         | 1.0±0.3 | D/A*, B†, C*, E†                         |
| CD3/HLA-DR    | 0.2±0.0                | 8.0±3.2  | 2.8±1.3  | 6.6±2.4         | 3.2±1.4 | B, D/A**                                 |
| CD3/IL-2R     | 0.0±0.0                | 0.4±0.1  | 0.3±0.8  | 0.4±0.2         | 0.2±0.1 | B, D/A**                                 |

\*Cells × 10<sup>4</sup>/mlBAL fluid; mean values ± SE from 5 healthy volunteers, 4 bronchiolitis obliterans organizing pneumonia (BOOP), 4 chronic eosinophilic pneumonia (CEP), 9 sarcoidosis, 10 diffuse panbronchiolitis (DPB).

\*p < 0.0001, \*\*p < 0.05, \*p < 0.01, †p < 0.001, †p < 0.005

incubated with 100 µl of peroxidase-conjugated antibody at room temperature for 4 h. The wells were subsequently washed 5 times with buffer A and incubated with 0.1 M acetate buffer, pH 5.5 containing tetramethylbenzidine for 10 min or until a suitable color develop. The reaction was stopped by adding 100 µl of 1 N HCl to each well. Plates were read the absorbance at 450 nm in an ELISA reader. The average level from duplicate assays, performed after a serial dilution of each sample, was obtained. The linear line was obtained at the range of 0 to 500 pg/ml of standard, and the detection limit was 7.8 pg/ml. A cross-reaction with other cytokines such as human granulocyte macrophage colony-stimulating factor, IL-4, interferon  $\gamma$ , tumor necrosis factor and mouse IL-5 was not observed.

### Statistical Analysis

All data are expressed as the mean ± standard error (SE). Statistical comparison was performed using one-way analysis of variance (ANOVA). Comparison between two parameters was made by using Spearman's analysis. A probability of less than 5% was considered to be significant.

## Results

*Eosinophils and lymphocyte subpopulations in bronchoalveolar lavage fluids.* The number of eosinophil was significantly higher in patients with CEP than in other lung diseases and healthy volunteers ( $p < 0.0001$ ), and tended to be high in BOOP compared to those in sarcoidosis and healthy volunteers (table 1). Quantification of lymphocyte subpopulations including CD3+, CD4+ and CD8+ was performed by direct immunofluorescence on BAL fluid cells. As shown in table 1, BOOP and sarcoidosis

showed a significant increase in CD3+ (vs healthy volunteers,  $p < 0.05$ ), and a increase in CD8+ cells of the former and in CD4+ cells of the latter. This results in a significantly increased CD4/CD8 ratio in sarcoidosis and a slightly decreased in BOOP. The CD4/CD8 ratios in other diseases showed intermediate values between BOOP and sarcoidosis, and revealed no significant differences compared with that in healthy volunteers. Activated T cells in BAL fluids were measured by the expression of HLA-DR and IL-2R. The relative and absolute numbers of HLA-DR bearing CD3+ cells were significantly increased in BOOP and sarcoidosis compared with those in healthy volunteers. Whereas in CEP and DPB the relative numbers showed significant increases (CEP:  $62.5 \pm 5.1\%$ ,  $p < 0.01$  vs healthy volunteers; DPB:  $49.4 \pm 6.4\%$ ,  $p < 0.05$  vs healthy volunteers), although much lower absolute numbers were observed than in BOOP and sarcoidosis. IL-2R bearing CD3+ cells revealed significantly increased absolute numbers in BOOP and sarcoidosis compared to healthy volunteers, but no significant differences were observed among the diseases.

*Interleukin 5 levels in bronchoalveolar lavage fluids.* Since IL-5 is known to be released by activated T cells and be associated with eosinophilia, IL-5 was measured by ELISA method in BAL fluid obtained from patients with interstitial lung diseases. As shown in Fig. 1, three (75%) of 4 patients with BOOP or CEP were measurable with an average value of  $79.7 \pm 46.4$  pg/ml or of  $68.7 \pm 29.9$  pg/ml and a significant higher level than those with healthy volunteers and DPB, whose IL-5 levels were all below detection limit. Furthermore, three CEP patients with the high level of IL-5 showed the marked elevation of eosinophils in BAL fluid (44.4% to 62.6%). The average level of IL-5 in sarcoidosis was much lower with  $25.1 \pm 17.0$  pg/ml than that in BOOP and CEP, and only 3 (33%) of 9 patients were positive.

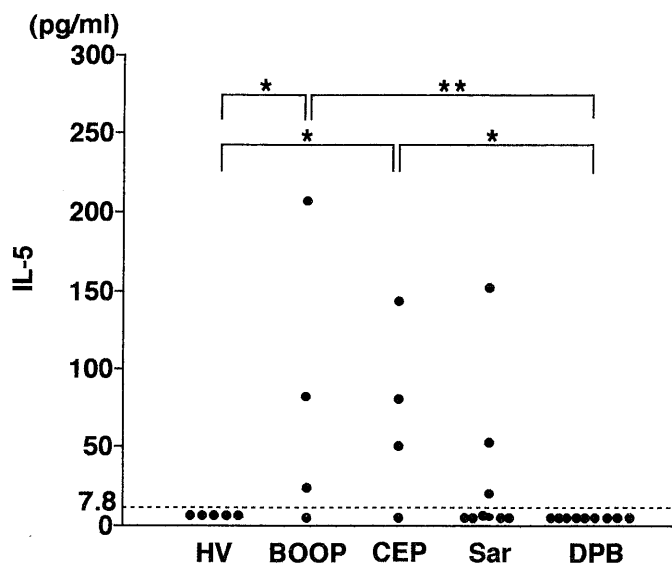


Fig. 1 Interleukin 5 levels in bronchoalveolar lavage fluid from patients with interstitial lung diseases compared to healthy volunteer (HV). The average level of IL-5 was  $79.7 \pm 46.4$  pg/ml in bronchiolitis obliterans organizing pneumonia (BOOP),  $68.7 \pm 29.9$  pg/ml in chronic eosinophilic pneumonia (CEP),  $25.1 \pm 17.0$  pg/ml in sarcoidosis (Sar) and 0 pg/ml in diffuse panbronchiolitis (DPB). \* $p < 0.05$ , \*\* $p < 0.01$ .

*Correlation between activated T cells and interleukin 5 levels in bronchoalveolar lavage fluids.* Fig. 2 shows a correlation between HLA-DR bearing CD3 + cells and IL-5 levels in BAL fluid from patients with interstitial lung diseases. A significant correlation of  $r = 0.581$  between these two parameters was found, especially in BOOP and CEP. Such correlation also exists between the number of CD4 + T cells and IL-5 levels, albeit weak and insignificant ( $r = 0.425$ ,  $p = 0.0758$ ; Fig. 3). No correlation between activated T cells and eosinophil count in BAL fluids was observed.

## Discussion

The present study demonstrates the increased number of lymphocytes in BOOP and of eosinophils in CEP in BAL fluid, and a decreased CD4/CD8 ratio in BAL fluid of BOOP as reported previously<sup>11-13</sup>. Additional two-color direct immunofluorescence analysis by flow cytometry revealed a marked increase of HLA-DR expression on BAL CD3+ cells in BOOP and CEP, indicating activation of T cell. Although another T cell activation marker, IL-2R (CD25) was also expressed on CD3+ cells in BAL fluids, the numbers was quite low compared to those of HLA-DR bearing CD3+ cells. It has been recently reported that CD25+ cells in BAL fluid from BOOP patients increased in Caucasian populations<sup>12</sup>. This discrepancy may be

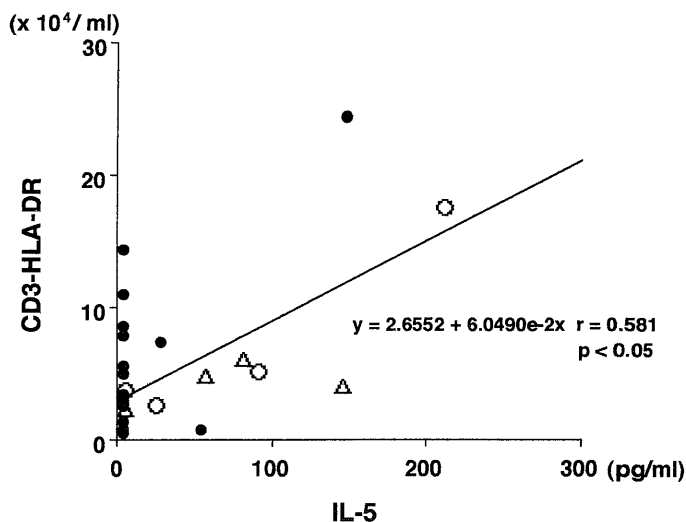


Fig. 2 Correlation between interleukin 5 levels and CD3-HLA-DR cells in bronchoalveolar lavage fluid from patients with interstitial lung diseases. Open circle and open triangle indicate patients with bronchiolitis obliterans organizing pneumonia and chronic eosinophilic pneumonia respectively, and closed circle indicates patients with other interstitial lung diseases and healthy volunteers.

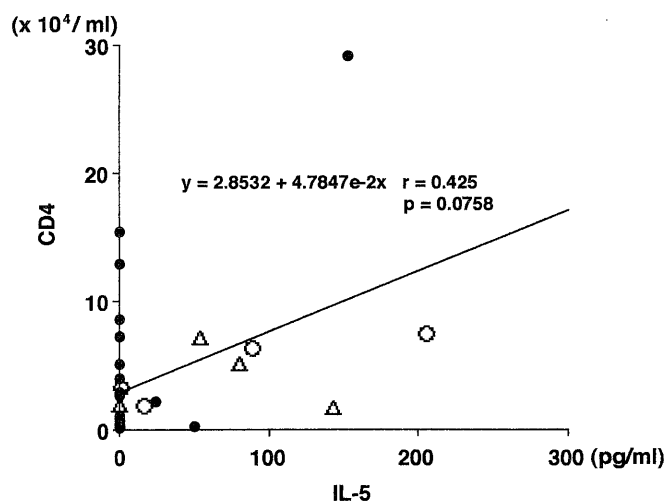


Fig. 3 Correlation between interleukin 5 levels and CD4 cells in bronchoalveolar lavage fluid from patients with interstitial lung diseases. Open circle and open triangle indicate patients with bronchiolitis obliterans organizing pneumonia and chronic eosinophilic pneumonia respectively, and closed circle indicates patients with other interstitial lung diseases and healthy volunteers.

explained by racial background<sup>14</sup> and our previous finding that no CD25 expression in CD3+ cells of BAL fluid was observed in Japanese sarcoidosis patients in contrast with high CD25 expression found in Caucasian<sup>9</sup>. Clinically, it is

difficult to differentiate BOOP from CEP, especially when presented cases are suspected of BOOP with multiple patchy migratory pulmonary involvements in the chest X-ray<sup>11)</sup>, and histologically, CEP shows a BOOP pattern<sup>15)</sup>. In our cases, lavage lymphocyte numbers were higher than lavage eosinophil numbers in patients with BOOP. On the other hand, lavage eosinophil numbers were higher than lavage lymphocyte numbers in 3 of 4 CEP patients. There was, however, one case of CEP with no prominent eosinophil numbers, which made it difficult to differentiate CEP from BOOP. These results suggest that a hyperimmune mechanism through activated T cells may be involved in pathogenesis in not only CEP, but also BOOP.

Activation of T cells results in secreting a variety of lymphokines, which play a vital role in the regulation and coordination of immune responses and inflammatory processes. It has been reported that T cells and secretion of lymphokines, mainly IL-5, play an important regulatory function toward eosinophils, which are thought to represent major proinflammatory effector cells in certain types of asthma<sup>4)</sup>. IL-5 is produced from helper T cells, CD4+ cells, especially Th 2 cells (memory T cells)<sup>5)</sup>, and is an important inflammatory cytokine which represents an eosinophil proliferation and differentiation factor and is chemotactic for eosinophils<sup>1-3)</sup>. In the present study, increased levels of IL-5 was found in BAL fluid from patients with BOOP and CEP. Furthermore, it is of interest that the expression of HLA-DR on CD3+ cells and IL-5 levels in BAL fluid is positively correlated, suggesting the important role of the activated T cells in the production or release of IL-5 in patients with BOOP and CEP. Additionally, IL-5 may be secreted from CD4+ cells in both diseases, as supported by a weak correlation between CD4+ cells and IL-5 levels in BAL fluid. Since CEP patients with the high level of IL-5 showed the marked elevation of eosinophils in BAL fluid, IL-5 may be related to eosinophilia in the lung of CEP, resulting in tissue injury of the airway and/or the alveoli through the production of granular constituents such as a major basic protein and eosinophilic cationic protein<sup>16-20)</sup>. Because of the poor relationship between eosinophilia and IL-5 level in BAL fluid from BOOP patients, further investigation will be required to determine whether IL-5 in BAL fluid is biologically active. Another explanation for this result is offered by the possible consideration that BOOP is one aspect in the course of CEP.

HLA-DR bearing T cells were also increased with sarcoidosis and DPB in the present study, and our previous study demonstrated the increased numbers of CD4+ CD29+ cells, helper-inducer (memory) T cell in peripheral blood and BAL fluid from patients with sarcoidosis<sup>9)</sup> and DPB (unpublished data). IL-5, however, was detectable in none of DPB patients of few individuals of sarcoidosis. These results lead us to the possibility that the state of T cell activation is different between BOOP or CEP and

sarcoidosis or DPB.

In conclusion, we have first reported that the activation of T cells and secretion of IL-5 might be involved in the pathogenetic process of same types of disease, BOOP and CEP, and our results indicate that lymphocyte-eosinophil interactions take place in the lung of CEP, but not in BOOP. Since IL-5 can prolong eosinophil survival *in vitro*<sup>4)</sup>, further investigation in the biological activity of the cytokine are required.

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