Differences in lymphocyte profile between BAL fluid and human lung tissue from patients with interstitial lung disease

F. FUJIMORI*, T. SHIMIZU*, T. TAKADA*, J. NARITA*, E. SUZUKI† and F. GEJYO*

Division of Respiratory Medicine, Graduate School of Medical and Dental Sciences, Niigata University; and †Department of General Medicine, Niigata University Medical and Dental Hospital, Japan

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ABSTRACT

Bronchoalveolar lavage (BAL) is a technique that samples the inflammatory cells from distal airways and alveoli; however, it is unclear whether or not cellular profiles in the BAL fluid reflect the cellular components of the lung parenchyma in interstitial lung disease (ILD). The aim of this study is to compare immunophenotypes of lymphocytes between BAL fluid and human lung tissue from patients with ILD. Fourteen consecutive patients with ILD who underwent BAL and surgical lung biopsy were enrolled. The diagnosis of ILD was confirmed by the presence of clinical symptoms and impaired respiratory function and on high-resolution computed tomography (CT) of the chest. Mononuclear cells in BAL were immunophenotyped for the expression of CD3, CD4, CD8, CD19, CD45, and CD103 by flow cytometry. Lung tissue obtained by surgical biopsy was digested with collagenase and then centrifuged to extract parenchymal cells. Isolated cells were also immunophenotyped for the same CD expression. Frequencies of positive cells were compared statistically between BAL and different lobes. Seven out of 14 patients were diagnosed clinically as suffering idiopathic interstitial pneumonia. Frequency of $CD19^+$ cells from BAL was significantly lower than that from the upper/middle lobes ($P<0.05$). Frequency of $CD103^+$ cells from BAL was significantly higher than that from the upper/middle lobes and the lower lobe ($P=0.01$ and $P<0.05$, respectively). Comparison between different lobes demonstrated that the frequency of $CD4^+$ cells from the upper/middle lobes was significantly lower than that from the lower lobe ($P<0.05$). The results suggest that lymphocyte immunophenotype profiles from BAL may not reflect those in the inflammatory tissue of ILD.

KEY WORDS: Bronchoalveolar lavage.
Flow cytometry.
Lung diseases, interstitial.
Lymphocytes.

Introduction

Interstitial lung disease (ILD) represents a diverse group of pathological conditions involving the lung parenchyma. At present, three methods are useful for detecting, staging and following the course of ILD, and these are physiological testing, high-resolution computed tomography (HRCT) scanning and bronchoalveolar lavage (BAL). The last is a relatively non-invasive technique that may provide important information about the immune and inflammatory mechanisms at the alveolar level.[1]

However, the role of BAL in defining the stage of disease and the assessment of disease progression or response to therapy remains poorly understood. Controversy exists about whether or not the BAL fluid cellular profile reflects the cellular composition of the lung parenchyma because inflammatory immune cells, secretions from the airway surface and the alveoli theoretically cannot be sampled by BAL. In addition, although the right middle lobe is lavaged most commonly, the lung parenchyma damage in ILD involves predominantly or exclusively the lower and peripheral lung zones.[2,3]

Surgical lung biopsy is the standard procedure for the diagnosis of ILD. Immunohistochemistry of lung biopsy tissue helps researchers to phenotype lymphoid infiltrates. Papiris *et al*. showed that in patients with idiopathic pulmonary fibrosis (IPF) T-lymphocyte subpopulations ($CD4^+$, $CD8^+$ and the $CD4^+$:$CD8^+$ ratio) recovered by BAL and evaluated using flow cytometry correlated closely with the corresponding values found in lung tissue evaluated by quantitative immunohistochemistry.[4] However, it is difficult to characterise all the cells in affected lung tissue because immunohistochemistry can evaluate only those cells present in a section of the lung tissue.

The authors have established a technique to isolate mononuclear cells from enzyme-digested human lung tissue and to characterise their immunophenotypes by flow cytometry.[5] To study whether or not there are any differences in lymphocyte immunophenotype between BAL and the lung interstitium, the technique is applied to lung biopsy tissue from patients with ILD in order to characterise the immunophenotype of isolated lymphocytes and compare them to those in the BAL fluid from the same patient. The subpopulations of lymphocytes found in different lobes of the affected lung are also compared.

Correspondence to: Toshinori Takada
Division of Respiratory Medicine, Graduate School of Medical and Dental Sciences, Niigata University, 1-757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan
Email: ttakada@med.niigata-u.ac.jp

Materials and methods

Study subjects

Fourteen consecutive patients with ILD who underwent BAL and surgical lung biopsy in Niigata University Medical and Dental Hospital from January 2002 to December 2005 were enrolled in the study. The diagnosis of ILD was confirmed by clinical symptoms, impaired respiratory function and HRCT of the chest. Those patients who showed specific findings such as lung cancer, sarcoidosis or Wegener's granulomatosis were excluded from the study. All subjects gave their written informed consent to participate in the study. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and the study was approved by the Committee of Ethics, Niigata University.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed according to procedures described previously.[6] Briefly, four boluses of 50-mL sterile 0.9% saline were introduced to the middle lobe via a bronchofibrescope under local anaesthesia. The BAL fluid was filtered through three layers of gauze and centrifuged to collect mononuclear cells. Total cell counts were performed using a haemocytometer, and cell differentials were determined after cytocentrifugation and May Grunwald-Giemsa staining. Collected mononuclear cells were resuspended in phosphate-buffered saline (PBS) at a concentration of 2 x 10^6/mL for flow cytometry.

Surgical lung biopsy and pathological study

All subjects underwent surgical lung biopsy by video-assisted thoracoscopic surgery. Two or three samples were taken from differently affected regions (as determined by HRCT). Specimens were divided into two pieces, one for diagnostic histopathology and one for mononuclear cell isolation. For histopathology, specimens were fixed in 4% formalin, processed to paraffin wax and stained by haematoxylin and eosin (H&E) and elastic van-Gieson methods. Pathological diagnosis was determined according to American Thoracic Society/European Respiratory Society (ATS/ERS) Consensus Classification.[7]

Isolation of mononuclear cells from lung biopsy tissue

Mononuclear cell isolation by enzyme digestion was performed according to procedures described previously.[5] Briefly, lung specimens were scraped extensively with a pair of sterilised scissors and the resulting homogenised suspension was digested with 0.5 mg/mL collagenase (Wako, Osaka, Japan) and 0.1 mg/mL trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA) in minimum essential medium (Gibco BRL, Bethesda, MD, USA) in a 37°C shaking water bath for 30 min. The digested lung tissue and supernatant were passed through a 200-gauge stainless steel mesh to remove cell clumps and undissociated tissue. The filtered suspension was centrifuged and the pellet was resuspended in PBS at a concentration of 2x10^6/mL prior to flow cytometry.

Flow cytometry

Cells suspended in PBS were stained with fluorescein isothiocyanate-, phycoerythrin-, or peridinin chlorophyll protein-conjugated monoclonal antibodies to CD3, CD4, CD8, CD19, and CD45 (Becton-Dickinson, San Jose, CA, USA) for 20 min at 4°C. In addition, expression of CD103 was evaluated on lymphocytes using a fluorescein-conjugated monoclonal antibody to CD103 (Becton-Dickinson) because this molecule is involved in various pathological

Table 1. Clinical data of ILD patients who underwent BAL and lung biopsy.

Case	Age (years)	Sex	Clinical diagnosis	Preceding therapy	Biopsy segment	Pathological diagnosis
1	50	F	IIP	none	R S2, S9	unclassifiable
2	72	F	IIP	none	*R S2, S6, S10	unclassifiable
3	51	F	IIP	none	R S4, S5	OP
4	50	F	IIP	none	L S4, S8	cellular NSIP
5	68	F	IIP	none	L S1+2, S6	unclassifiable
6	65	F	IIP	none	L S1+2, S8	fibrosing NSIP
7	60	F	IIP	none	L S1+2, S8	cellular NSIP
8	63	M	CVD-ILD	none	R S4, S8	unclassifiable
9	72	F	CVD-ILD	none	R S2, S6	unclassifiable
10	43	F	RA/DM-ILD	PSL 5mg/day MTX 6mg/week	L S1+2, S8	unclassifiable
11	44	F	MCTD-ILD	PSL 2mg/day	R S2, S6	cellular NSIP
12	49	F	CVD-ILD	none	*R S2, S6, S9	unclassifiable
13	70	M	CVD-ILD	none	R S2, S9	unclassifiable
14	69	M	Sjs-ILD	none	R S4, S10	unclassifiable

CVD: collagen vascular disease, DM: dermatomyositis, IIP: idiopathic interstitial pneumonia, MCTD: mixed connective tissue disease, MTX: methotrexate, NSIP: non-specific interstitial pneumonia, OP: organising pneumonia, PSL: prednisolone, RA: rheumatoid arthritis, Sjs: Sjögren syndrome.

*Mononuclear cells were isolated from S2 and S10 (case 2) and S2 and S9 (case 12).

conditions.[8–12] Stained cells were analysed by flow cytometry (FACScan, Becton-Dickinson) using a Cell Quest program.

Differentiation of lymphocytes was determined using flow cytometric analysis of light scatter characteristics relating to size and granulation, and fluorescence gating with anti-CD45 antibody. Three-colour flow cytometry was performed to calculate the percentages of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD103^+$ cells in a subset of $CD45^+$ lymphocytes.

Statistical analysis

Comparison of paired non-parametric quantitative data between the two groups was made by a Wilcoxon's signed rank sum test. $P<0.05$ was considered significant.

Results

Seven patients were diagnosed with idiopathic interstitial pneumonia (IIP) because of their occupational history, physical findings and laboratory data, which did not suggest ILD. Three patients were diagnosed with ILD associated with rheumatoid arthritis/dermatomyositis, mixed connective tissue disease or Sjögren's syndrome, according to their clinical symptoms, laboratory data and the presence of autoimmune antibodies.

Although four patients with ILD did not fulfil the diagnostic criteria for collagen vascular disease (CVD), they were thought to be suffering from CVD-ILD because of the presence of various symptoms such as arthritis, skin rash and autoimmune antibodies, which suggested underlying CVD. Although 12 patients had not taken immunosuppressive agents, two patients with CVD-ILD had been treated with oral prednisolone (2–5 mg/day) and methotrexate (6 mg/week). These data are shown in Table 1.

Two or three biopsies were taken from multiple sites in the same lung. Occasionally, apparently normal tissue (on HRCT) was taken, but areas of end-stage scarring or honeycomb change were avoided (Table 1).[13] In a patient with organising pneumonia (OP, case 3), both biopsies were taken from the middle lobe, but one appeared normal macroscopically. Of the seven patients with IIP, one had OP, one had non-specific interstitial pneumonia (NSIP) with a fibrosing pattern, two had cellular NSIP (Fig. 1) and one patient with CVD-ILD was diagnosed as NSIP with a cellular pattern. The four IIP and six CVD-ILD patients were not classified into any of the seven entities covered by the ATS/ERS consensus criteria (Table 1, Fig 2).[7]

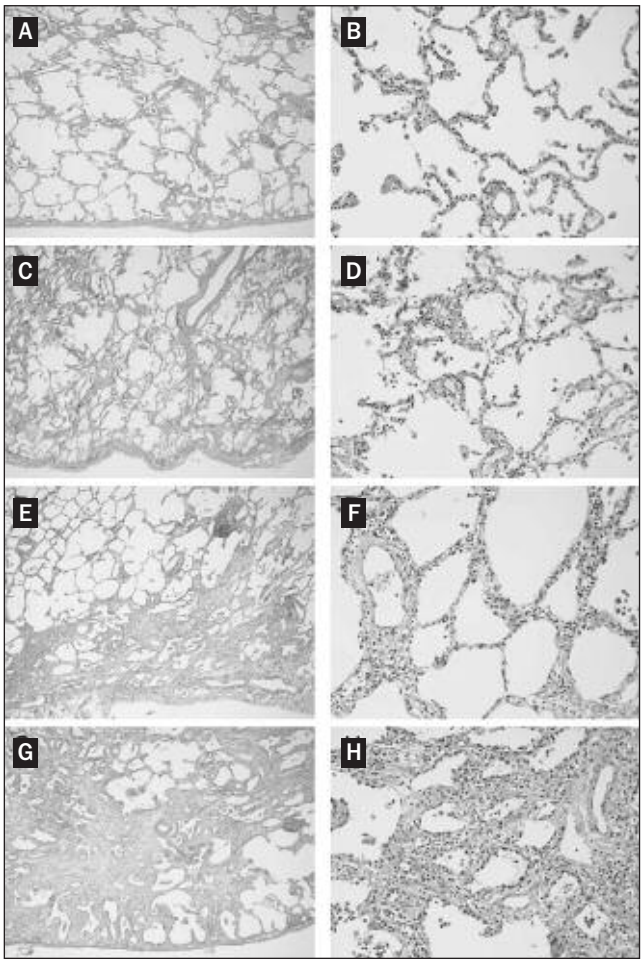

Fig. 1. Light microscopy findings of surgically obtained lung tissue. Moderate interstitial chronic inflammation without dense interstitial fibrosis or organising pneumonia is shown in the left upper lobe, S1+2 (A, B) and left lower lobe, S8 (C, D) of a patient with non-specific interstitial pneumonia (NSIP), cellular pattern (case 7). Loose interstitial fibrosis lacking the temporal heterogeneity pattern is observed in the left upper lobe, S1+2 (E, F) and left lower lobe, S8 (G, H) of a patient with NSIP, fibrosing pattern (case 6). Interstitial inflammation and fibrosis are more severe in the lower lobe in both cases (original magnification x40 [A, C, E, and G] and x200 [B, D, F and H]).

Table 2. Comparison of frequency of mononuclear cells between BAL and lung biopsy.

Monoclonal antibodies	BAL	Upper/middle lobe	Lower lobe
CD3	80.9±16.7	73.0±15.2	77.3±8.7
CD4	35.5±23.3	29.1±12.2*	32.2±12.2
CD8	47.8±24.7	45.4±18.0	44.1±16.3
CD4/8 ratio	1.37±1.65	0.85±0.69	0.88±0.56
CD19	2.14±3.34†	4.44±3.45	4.28±2.98
CD103	46.6±21.0‡§	31.1±20.3	32.6±18.0

Mean±SD shown.

*$P<0.05$ against lower lobe, †$P<0.05$ and ‡$P=0.01$ against upper/middle lobe, and §$P<0.05$ against lower lobe.

Mononuclear cells from BAL fluid and enzyme-digested lung biopsy material were analysed by flow cytometry to evaluate the expression of the surface antigens CD3, CD19, CD4, CD8 and CD103 (Fig. 3). The frequency of $CD19^+$ cells in BAL was lower than that found in the upper or middle lobes, but this disparity was not apparent between the upper/middle and lower lobes. The frequency of $CD103^+$ cells in BAL was significantly higher than that in the upper/middle lobes or the lower lobe ($P=0.01$ and $P<0.05$, respectively). Comparisons between different lobes demonstrated that the frequency of $CD4^+$ cells in the upper/middle lobes was lower than that in the lower lobe. These data were summarised in Table 2.

Discussion

It is unclear whether or not the cellular profile in BAL fluid reflects the cellular composition of the lung parenchyma, and this has been the source of some debate. In the present study, mononuclear cells extracted from human lung biopsy tissue were compared with those found in BAL fluid in patients with ILD. Statistical comparison of data revealed differences in the frequency of $CD19^+$ cells between BAL and the upper/middle lobes, and in $CD103^+$ cells between BAL and either lobe. A difference in frequency of $CD4^+$ cells between the upper/middle and lower lobes was demonstrated.

Although BAL is usually non-specific and its ability to predict underlying pathology, stage disease or predict response to therapy is limited, it has been used to study the mechanisms of lung injury, inflammation, repair and fibrosis in many pulmonary diseases.[1,14] In ILD, the right middle lobe is commonly lavaged because the anatomy favours maximal recovery of fluid and cells from these sites when the patient is lying in a supine position. Lavage at that site is thought to provide a representative picture of the inflammatory and immune processes in the alveoli.[15]

Lung parenchymal damage in ILD involves predominantly or exclusively the lower and peripheral lung zones.[2,3] Therefore, information obtained from the right middle lobe by BAL may not reflect lung parenchymal damage in ILD.

CD103, the integrin αEβ7, has a narrow and highly specialised pattern of expression and is restricted largely to $CD8^+$ cells. $CD8^+$ $CD103^+$ cells are present in or adjacent to mucosal epithelial cells *in vivo*.[16] CD103 plays a role in tissue-specific retention of activated peripheral blood lymphocytes by interacting with E-cadherin. Thus, it is reasonable to suppose that $CD103^+$ cell frequency would be higher in BAL than in isolated cells from the lung interstitium because most cells sampled by BAL should be located in the alveolar space and on the airway surface.

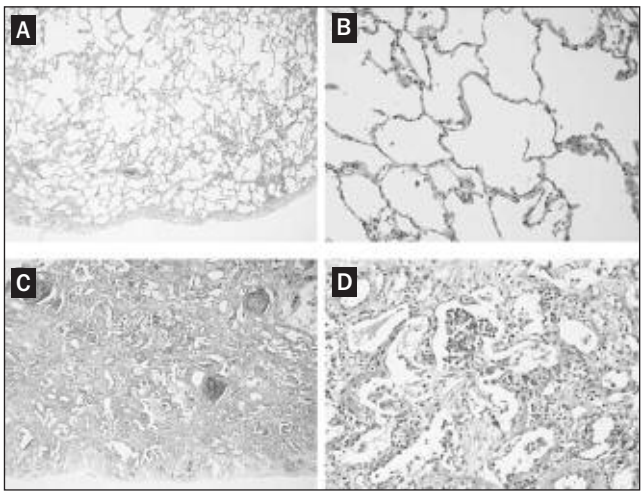

Fig. 2. Light microscopy findings of unclassifiable ILD from case 12. Mild interstitial inflammatory regions are present in almost normal alveolar walls in the right upper lobe, S2 (A, B). In contrast to the right upper lobe, loose interstitial fibrosis without the temporal heterogeneity pattern is mixed with loose plugs of connective tissue within a bronchiole and the adjacent alveolar duct and alveolar spaces and lymph follicles in the right lower lobe, S9 (C, D) (original magnification x40 [A, C] and x200 [B, D]).

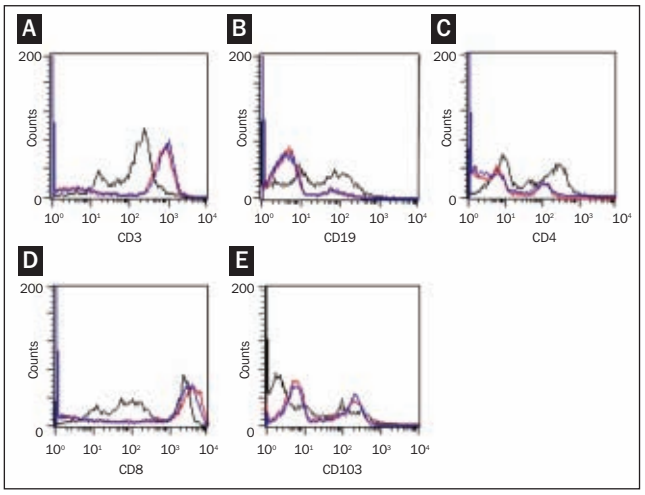

Fig. 3. Representative histograms of immunopositive lymphocytes carrying each CD from BAL (black), upper lobe (S2, blue) and lower lobe (S10, red) of case 2. Mononuclear cells from BAL or either lobe were stained for surface expression with anti-CD3 (A), CD19 (B), CD4 (C), CD8 (D) and CD103 (E) followed by flow cytometry analysis. The blue and red lines are almost parallel, but the black line shows a distinctive curve from the other two lines. Immunopositive cells gated with anti-CD45 antibody were counted for each CD antigen. See the image in colour at www.bjbs-online.org

It is reported that $CD8^+$ $CD103^+$ lymphocytes are involved in the development of pathological conditions such as systemic lupus erythematosus, Sjögren's syndrome, kidney allograft rejection and psoriasis.[8–12] Although T lymphocytes expressing CD103 have been reported in BAL fluid,[17] their involvement in developing ILD remains unclear. The inflammation in ILD often involves not only the alveolar walls and epithelium but also the capillary endothelium, the spaces between these structures, and the perivascular and lymphatic tissues. However, cells located in the interstitium around the alveolar space or airway surface cannot be sampled by conventional BAL. Isolation and immunohistochemical characterisation of cells present in this interstitial zone may be useful in the accurate evaluation of the inflammatory process in ILD.

Surgical lung biopsy is the most effective method for confirming the diagnosis and assessing disease activity in diffuse parenchymal lung diseases, particularly ILD, as histopathological studies may identify a more treatable process than originally suspected. It is recommended that biopsies be taken from multiple lobes because a biopsy often samples only a portion of a particular disease process, and this cannot be extrapolated to all regions.[18] Multiple biopsies from multiple sites may yield prognostic information about active inflammation and the extent of fibrosis.

In the present study, although the histopathology of multiple biopsies revealed variations in cell distribution and disease severity, only a difference in $CD4^+$ cell frequency was identified between multiple sites. The results suggest that the inflammatory process in ILD may be similar throughout the lung, and that only the degree of progression may be different between various regions.

The present study had several limitations. Nine out of the 14 patients who had ILD were diagnosed as unclassifiable ILD; however, the ATS/ERS consensus classification includes

seven clinical/radiological/pathological entities.[7] Although the nine patients seemed, on clinical and radiological grounds, to have NSIP or COP, the histopathology was not classified (e.g., case 12 [Fig. 2]). Two out of 14 patients in the study had received treatment (e.g., prednisolone); however, the presence of unclassifiable ILD or previous therapy may not affect the conclusions drawn from this work because phenotypic analysis was performed on BAL and lung tissue from the same patients.

As the lung tissue was digested using collagenase, the recovered cells should contain alveolar epithelial cells, peripheral airway epithelial cells and endothelial cells, in addition to mononuclear cells. Thus, total cell counts per gram of lung tissue could have been calculated, and comparisons between different regions in the same lung may have yielded information on the severity of inflammation in a particular region.

In conclusion, the results of the present study suggest that lymphocyte immunophenotype profiles obtained from BAL fluid may not reflect those in the inflammatory lung tissue of ILD. Thus, it is necessary to investigate further the limitations of BAL and to understand the exact mechanisms of inflammation in ILD using other available methods.

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