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Bronchoalveolar Lavage Fluid Analysis Provides Diagnostic Information on Pulmonary Langerhans Cell Histiocytosis

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Abstract

Histiocytes of Langerhans cell type are recovered from the bronchoalveolar lavage fluid (BALF) of patients with interstitial lung diseases in a nonspecific manner. Langerhans cells (LCs) can be identified through immunostaining for S-100, CD1a, and, more specifically, langerin. To evaluate the diagnostic value of BALF in pulmonary Langerhans cell histiocytosis (PLCH), we performed a retrospective clinicopathological study of 5 patients with biopsy-confirmed PLCH or Hand-Schüller-Christian disease involving the lung. As a control study, we examined BALF cells from 23 patients with various diseases, including sarcoidosis, hypersensitivity pneumonitis, collagen vascular disease, idiopathic pulmonary fibrosis, and adenocarcinoma of the lung. Cytospins obtained from BALF were stained with Giemsa or Papanicoloau and others were immunostained. In general, cytospins showed a monomorphous and dispersed cell population containing mononucleated or binucleated and occasionally multinucleated histiocytes. LCs recovered from BALF were characterized by clear and velvety cytoplasm; oval or kidney-shaped, vesicular nuclei with irregular shapes; nucleoli; and frequent grooves and indentations. Radiography and high-resolution computed tomography showed multiple bilateral nodular or cystic lesions in the middle and upper lung zones. The mean percentage of LCs in 9 lavages from the 5 patients was 8.0%, whereas that from the control group was only 0.3% (maximum, 1.6%). The percentage of cells positive for S-100 or CD1a was comparable to the percentage of Langerhans-like histiocytes stained with Giemsa stain. The present results indicate that the survey of LCs in BALF with the aid of immunocytochemical evaluation and corresponding clinical data could play a critical role in establishing the diagnosis of PLCH, thus providing a less invasive approach than lung biopsy, which carries a risk of complications.
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Key words: lung, bronchoalveolar lavage, Langerhans cell histiocytosis, S-100, langerin

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Introduction

Langerhans cells (LCs), a type of histiocyte, are found in the epidermis of healthy skin. LCs play a distinct role in presenting antigens in the skin and are characterized by the cytoplasmic inclusions called Birbeck granules which are detectable at the ultrastructural level.

LCs are believed to be present in small numbers in the airways of the healthy lung but tend to increase in lung tissue showing higher degrees of fibrosis, such as in interstitial lung diseases. The function of LCs in the lung remains obscure. The routine diagnosis of pulmonary Langerhans cell histiocytosis (PLCH) is based on biopsy. Evidence of Birbeck granules in the cytoplasm is required for a definitive diagnosis; nevertheless several clues supporting the diagnosis, such as compatible chest radiographs, history of heavy smoking, and minimal or no symptoms, also exist.

Electron microscopic observation[1] indicates that PLCH is strongly related to cigarette smoking and is characterized by the proliferation of LCs and their infiltration in organ systems[2]. As a less invasive approach, the cytological analysis of BALF is recommended, with a percentage of LCs greater than 5% being an important diagnostic criterion for PLCH[3–6].

Normal LCs are present only within the bronchial and bronchiolar epithelia [7,8]. Similarly, the granulomatous lesions in PLCH are highly bronchiolocentric, suggesting that the epithelial microenvironment remains an important determinant for the accumulation of LCs in this disease[9,10]. Because of the prominent effects of heavy smoking on the bronchiolar epithelium and the strong association between PLCH and smoking, the possibility that smoking-induced changes in the epithelium promotes accumulation of LCs is attractive. In addition, epithelial cells can produce a variety of cytokines, including factors that influence the proliferation, survival, and differentiation of LCs[7,11].

The bronchial tree is normal on gross examination or shows only nonspecific inflammation related to smoking. Bronchial mucosal biopsy is not helpful in the diagnosis of PLCH but is useful for ruling out other diagnoses, particularly in patients with atypical manifestations. Transbronchial biopsy may confirm the diagnosis of PLCH, if multiple specimens are obtained and examined extensively, with immunohistochemical techniques if necessary[12]. Studies from the United States, where transbronchial biopsy is more widely used in this setting, have found diagnostic yields ranging only 10% to 40%, reflecting the focal distribution of the parenchymatous lesions[12–14]. In addition, the risk of pneumothorax is probably increased in patients with pulmonary cysts.

In the present study, we examined the value of BALF analysis for diagnosing PLCH and avoiding multiple lung biopsies and associated complications.

Materials and Methods

During the past 24 years, we have examined the BALF of more than 2,500 persons, including healthy control subjects with no respiratory disease (**Table 1**). This cohort included patients with diagnosis ranging from interstitial lung diseases of various subtypes to sarcoidosis, tuberculosis, and pneumonias. In the present study, we focused on patients with biopsy-confirmed PLCH. In our cohort, 20 patients had received a diagnosis of PLCH. We performed a retrospective clinicopathological study and reviewed the hospital records of these patients. For 5 patients with a biopsy-confirmed diagnosis of PLCH or Hand-Schüller-Christian disease with lung involvement[15,16] (courtesy of Dr. Atsuyuki Kurashima, National Tokyo Hospital) full clinicopathological data were available for comprehensive analysis. These 5 patients comprised the population for the present study. All patients were evaluated with special references to age; sex; occupation; smoking history; symptoms; results of lung imaging studies, including high-resolution computed tomography (HRCT); histopathologic examination, including staining with hematoxylin and eosin, Giemsa, Papanicoloau, and periodic acid methenamine silver (PAM) stains and immunocytochemical staining for S-100 (Dako A/S, Denmark), CD1a (Immunotech Inc., Marseille,

Table 1 Clinical diagnosis of the patients (Nippon Medical School-affiliated hospitals, April 1984 – November 2008)

Clinical diagnosis	Patients (%)
Interstitial lung diseases	936 (36.9)
IPF/UIP	108 (4.3)
NSIP	43 (1.7)
COP/BOOP	126 (5.0)
CVD-IP	168 (6.6)
COPD	117 (4.6)
IIPs	374 (14.7)
Sarcoidosis	295 (11.6)
Radiographic abnormal shadows	249 (9.8)
Eosinophilic pneumonia	135 (5.3)
Leukemia, lymphoma and others	113 (4.5)
Hypersinsitivity pneumonitis	125 (4.9)
Pneumoconiosis (Asbestosis, Coal workers)	95 (3.7)
Malignant tumor (Primary or metastat. Cancer)	87 (3.7)
Tuberculosis	73 (2.9)
ARDS/AIP	64 (2.5)
Fungus, P.Carinii	49 (1.9)
Bloody sputum	35 (1.4)
Pulmonary Langerhans cell histiocytosis	20 (0.8)
Alveolar proteinosis	14 (0.6)
Mycobacterium infection	11 (0.4)
Wegener's granulomatosis	4 (0.2)
The others	233 (9.2)
Total	2,538

IPF: idiopathic pulmonary fibrosis
UIP: usual interstitial pneumonia
NSIP: non-specific interstitial pneumonia
COP: cryptogenic organizing pneumonia
COPD: Chronic obstructive pulmonary disease
BOOP: bronchiolitis obliterans organizing pneumonia
CVD-IP: collagen vascular disease-related interstitial pneumonia
ARDS: acute respiratory distress syndrome
AIP: acute interstitial pneumonia.

France), and langerin (Abcam plc, Cambridge, UK) as reported previously[17]; and, finally, BALF cell analysis.

BALF was obtained after premedication with atropine and hydroxyzine. A bronchoscope was inserted under local anesthesia with lidocaine and wedged at the orifice of the objective subsegment of a lobe. The lavage was performed with infusion of 30 to 50 mL of sterile 0.9% saline for 3 times at the same site. The recovery rate of lavage fluid and the mean total cell count were determined. Then, the cytospin cells were stained with the May-Grünwald Giemsa and Papanicolaou methods. The differential cell counts were performed under a light microscope by counting more than 500 cells, and the means ± SE of measurements were determined. The statistical significance of data was assessed with the paired-sample *t*-test, and differences with a $p<0.05$ were considered significant. **Table 2** summarizes the clinical features of the patients. There were 3 men (age range: 23–58 years) and 2 women (age range: 18–42 years). All patients were smokers, and 2 were heavy smokers. Symptoms included cough, weight loss, and dyspnea.

Table 2 Clinical Features of Pulmonary Langerhans Cell Histiocytosis Patients

Patient	Sex	Age	Smoking*	Occupation	Symptoms
1	M	23	5	office work	cough, weight loss
2	M	45	18	construction work	cough
3	M	58	6	salesman	dyspnea
4	F	42	40	saleswoman	cough, dyspnea
5	F	18	2	none	cough

*: pack-years

Table 3 High Resolution Computerized Tomography (HRCT) Characteristics of Pulmonary Langerhans Cell Histiocytosis Cases

Patient	Cysts		Nodules		Distribution
	Upper Lobe	Lower Lobe	Upper Lobe	Lower Lobe	
1	(3+)*	(1+)	0	0	peripheral two third dominant
2	(2+)	(1+)	(1+)	(1+)	peripheral one third dominant with pleural involvement
3	0	(2+)	0	0	severe reticulation in upper lobes with pleural thickness
4	(2+)	0	(1+)	0	peripheral two third dominant
5	(3+)	(2+)	0	0	peripheral one third dominant

*: Degrees of each abnormality: 0; none, (1+); mild, (2+); moderate, (3+); severe

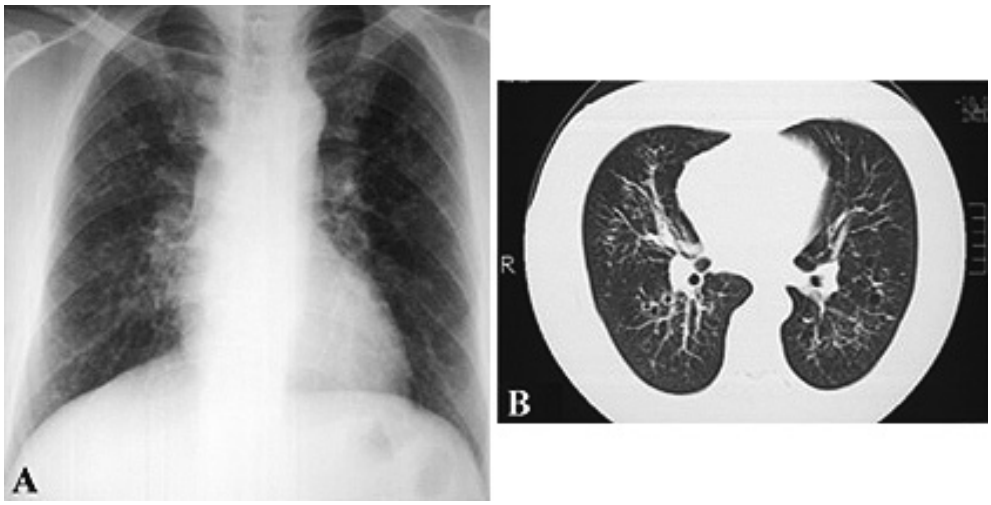

Fig. 1 Representative chest radiograph (A) and HRCT (B) of a case of PLCH shows multiple nodular and cystic changes predominantly in the middle and upper lobes of the lung.

Results

Diagnostic Imaging of the Lung

Figure 1 shows representative chest radiograph and HRCT and **Table 3** summarizes the findings from HRCT of the patients as characterized by interstitial lesions such as annular lesions, mainly in the upper lung zone. Frequency of cystic and nodular lesions mainly in the bronchiolar region was assessed at 4 grades: 0, none; 1+, slight; 2+, moderate; and 3+, severe[18].

The most common findings of PLCH included nodular and cystic changes, which occurred mostly in the middle and upper lobes. The most frequent abnormality was the presence of lung cysts, which were typically smaller than 20 mm in diameter and had thin walls on HRCT. Here, the frequency of cystic change reflects the timing of the HRCT over the course of the illness. As has been reported

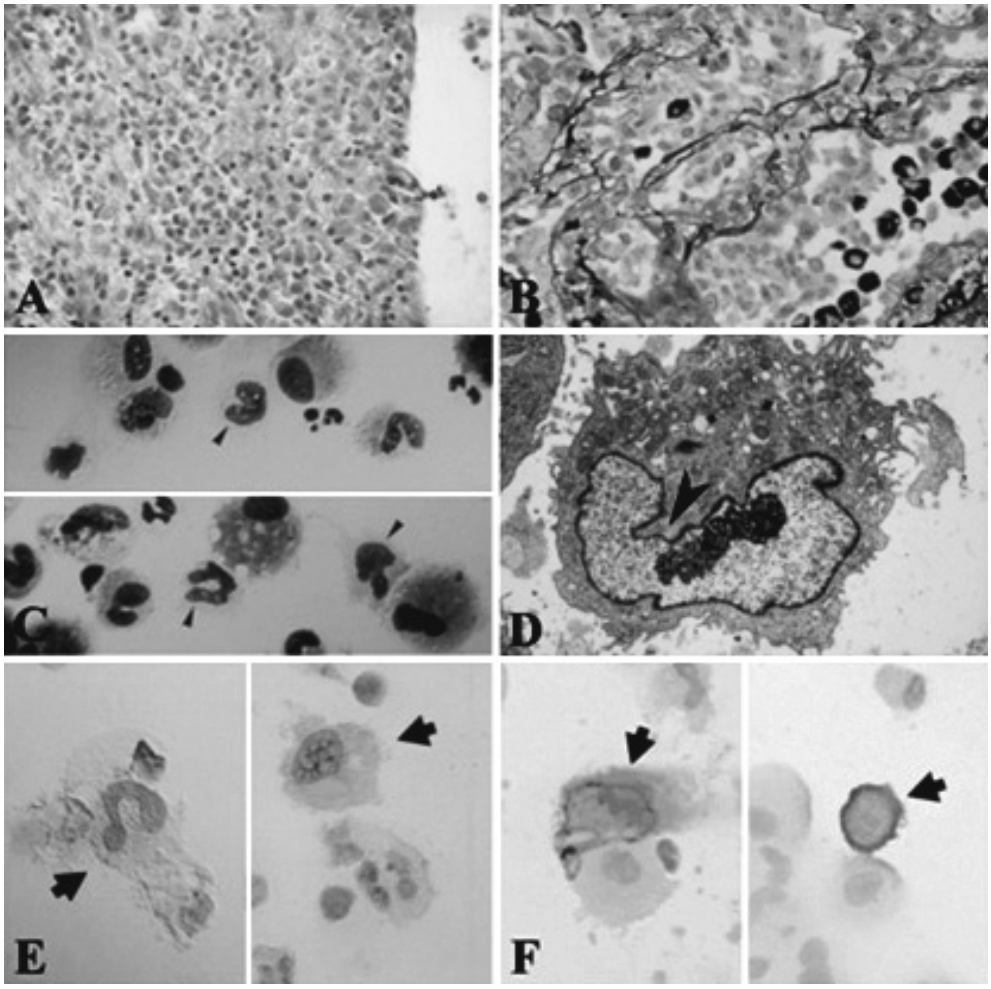

Fig. 2 Histopathological and cytological features of PLCH in tissue sections stained with (A) hematoxylin and eosin and (B) PAM. (C) On light microscopy with Giemsa staining **(arrowheads)** and (D) electron microscopy LCs show a semicircular or broad bean-shaped nuclear morphology with a deep, sharp depression **(arrowhead)**. (E) Representative immunocytochemical staining for S-100 protein **(arrows)** and (F) langerin showing positive reactions in the cytoplasm and at the cell surface of LCs **(arrows)**.

previously, in the early stages of PLCH, the most common findings are that of nodular changes, whereas in the later stages of disease cystic change and fibrosis tend to predominate[19]. In 4 patients, cysts were seen in the upper lung zone, and in 3 of these 4 patients cysts were also seen in the lower lung zone. No patients had cysts only in the lower lung zone. The maximum diameter of all cysts was 18 mm, and the cyst walls were thin and appeared hypertrophic and irregular in some areas. In 2 patients, cysts were complicated by nodular lesions. These nodular lesions were 2 to 10 mm in diameter and were seen in the peripheral regions, rather than in the center of the pulmonary hilum. In 1 patient, nodular lesions were adjacent to the pleura. Unlike the other patients, patient 3 lacked nodular lesions, and irregular and marked fibrotic lesions were seen in the upper lung zone, accompanied by pleural thickening.

Histopathologic Examination of Lung Biopsies

Specimens obtained at transbronchial lung biopsy or open-chest lung biopsy contained areas of granuloma, scar-like changes **(Fig. 2A)**, and some normal peripheral lung tissues. In PAM-stained tissue specimens **(Fig. 2B)**, LCs formed granulomas containing histiocytes with bright reticula, and black alveolar macrophages with many engulfed granules reacting to PAM stain were seen inside the granulomas. LCs showed semicircular nuclei with

Table 4 Analysis of Bronchoalveolar Lavage Fluid (BALF) in Pulmonary Langerhans Cell Histiocytosis Patients

Patient	Mφ (%)	Ly (%)	Neu (%)	Eo (%)	LC (%)	Giemsa stain*
1	52	11	12	16	9	(3+)
	77	5	1	1	16	(4+)
	69	12	0	10	9	(3+)
2	79	10	6	1	4	(1+)
	89	3	2	1	5	(2+)
3	75	8	3	2	11	(4+)
	87	8	1	1	3	(1+)
4	80	10	0	1	9	(3+)
5	69	12	5	10	6	(2+)
Mean ± SE	75.2±3.7	8.8±1.0	3.3±1.3	4.8±2.0	8.0±1.3	N/A

Mφ: Macrophage, Ly: Lymphocyte, Neu: Neutrophil, Eo: Eosinophil, LC: Langerhans cell, N/A: Not applicable.
*: Rates of the specific histiocytes in Giemsa stain estimated as: (1+); <5%, (2+); <7%, (3+); <10%, (4+); >10%

well-developed nucleoli and possessed a narrow and deep depression, matching the morphology of the specific histiocytes seen in Giemsa-stained specimens **(Fig. 2C)** and with electron microscopy **(Fig. 2D)**. LCs positive for S-100, CD1a, or langerin were also confirmed **(Fig. 2E, F)**. The degree of reactivity to the anti-S-100 antibody varied among histiocytes. Eosinophil infiltration was sometimes severe, enough to indicate a tumor, but was generally sporadic and irregular in-side and outside the granulomas. LCs composing the granuloma congregated densely.

Analysis of BALF

Table 4 shows the results of BALF analysis performed on a total of 9 lavages obtained from the 5 patients. The recovery rate of lavage fluid was 42.1%, with a mean total cell count of 4.06×10^5 /mL. Mean cell fraction rates for macrophages, lymphocytes, neutrophils and eosinophils were 75.2%, 8.8%, 3.3%, and 4.8%, respectively. Eosinophil rates varied from 1% to 16%, and eosinophils were consistently recovered in the BALF. Such fluctuations were also seen with lymphocytes and neutrophils, and no defined tendencies were seen.

Specific histiocytes that were the target of our observations displayed a semicircular or broad bean-shaped nuclear morphology, with a deep and sharp depression **(Fig. 2C, D)**. While these cells resembled segmented leukocytes, the depression was in fact equivalent to a waist. Histiocytes were chromatin-poor with well-developed nucleoli, and clearly differed from leukocytes morphologically. In S-100-positive cells that appeared to be LCs **(Fig. 2E)**, the characteristics, such as nucleus, cytoplasm, and cytoplasmic projections, matched well. Reticula were bright and velvety and larger than monocytes, appearing as irregular circles with occasional pseudopod-like cell projections. All 5 patients with confirmed diagnosis were smokers, and because macrophages had engulfed many dark-purplish granules, the cytoplasm tended to be dark **(Fig. 2C)**. These specific histiocytes could be relatively easily differentiated, given a lack of engulfed granules and the presence of bright and notable reticula. These cells were mostly solitary, but in some cases, several cells were sporadically seen within a limited field of view. The rate of S-100-positive cells was usually similar to the rate of histiocytes in Giemsa-stained specimens, and among the 500 cells observed the mean rate was 8.00% ±4.03% (range: 3%−16%). In patient 3, in whom a chronic scarred lesion was the main finding, the rate of LCs was as high as 11%. In controls subjects with various interstitial lung diseases and lung cancer **(Fig. 3)**, the rate of specific histiocytes was as high as 1.6% and was markedly lower than in patients with PLCH ($p<0.001$).

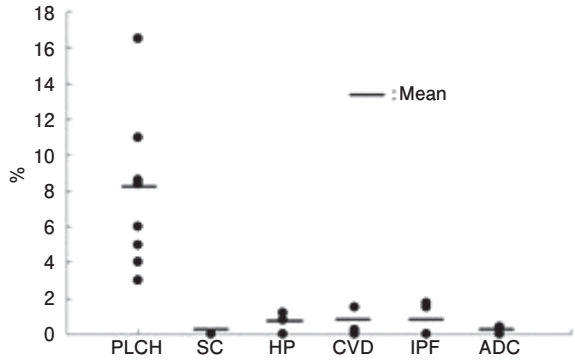

Fig. 3 The rate of LCs in the representative lung diseases shows that the mean LC rate in PLCH was 8.00 versus 1.6% in the control group. PLCH: pulmonary Langerhans cell histiocytosis; SC: sarcoidosis; HP: hypersensitivity pneumonitis; CVD: collagen vascular disease-related interstitial pneumonia; IPF: idiopathic pulmonary fibrosis; ADC: adenocarcinoma of lung.

Discussion

LCs, a type of middle-sized histiocyte of hematopoietic origin, are widely distributed through normal skin, oral and eye mucosa, airways, lungs, urinary tract, vagina, and other organs. LCs play important roles as antigen-presenting cells, as do macrophages and histiocytes, in physiological and pathological conditions. They proliferate in a form of granulomatous lesion called Langerhans cell granulomatosis or Langerhans cell histiocytosis, which was previously called eosinophilic granuloma (this term was long applied in Japan), and systemic organs are involved, as in Hand-Schüller-Christian disease[15,16] and systemic histiocytosis X.

In patients with a history of heavy smoking, the majority of macrophages in BALF contained a large number of pigmented particles in the cytoplasm. Among these macrophages, the smaller ones showed clear cytoplasm with no pigments at all. Monocytes had clear cytoplasm and a single large nucleus that was indented, giving it a horseshoe appearance.

In the present study, 4 of the 5 patients had mild physical findings, and plain chest radiography and computed tomography showed multiple cystic and nodular lesions in the upper and middle lung fields, as in previous reports[9,20,21]. Furthermore, lung biopsy findings were consistent with Langerhans-cell granulomatosis, and the diagnosis of PLCH was thus reasonable[11,13,21]. The remaining patient (patient 3) had systemic Langerhans-cell granulomatosis and lung complications (Hand-Schüller-Christian disease)[16].

In the present series, BAL was performed a total of 9 times. The rates of macrophages, lymphocytes and eosinophils recovered with BAL varied greatly (**Table 4**), and, in particular, the mean rate of eosinophils was approximately 5% (range: 1%–16%). This value was clearly lower than those in eosinophilic pneumonia accompanied by BALF-confirmed eosinophilia[22] (acute: 42%; chronic: 35%), bronchial asthma (28%), and pneumocystis pneumonia (20%). However, the above value was slightly higher than that in bronchiolitis obliterans organizing pneumonia (3.1%) or usual interstitial pneumonia (2.7%). The eosinophil fraction rate for the present disease thus lacks specificity and serves only as a reference for differential diagnosis.

Cells that were judged to be specific histiocytes on the basis of morphological characteristics with Giemsa staining (**Fig. 2C**) matched the cellular morphology of S-100-positive cells as seen in various diseases, including the present diseases. Compared with inflammatory cells in BALF, specific histiocytes clearly differ in size, presence of reticular granules, reticular and nuclear morphologies, and histochemical reactions[13,18,23]. Cells having these morphological characteristics were also shown to be OKT6-positive along the membrane and possessed Birbeck granules[24], and the assumption that these cells represented LCs appeared reasonable. However, the present study only compared and examined cellular morphologies. Whether Birbeck granules can be seen in all specific histiocytes remains unclear. LCs themselves also reportedly lose specific granules in a state of activate antigen delivery, suggesting that the presence of Birbeck granules is not the only means of differentiation[18].

In the present series of PLCH, the mean rate of specific histiocytes in BALF was 8.00% (range, 3%–16%). Previous studies have reported a recovery rate of 5.29%[9,18], and other studies have documented high rates of 5% or more[25]. These studies stated that the recovery rate would offer an important guide for

observing the disease course. As seen in patients 1, 2, and 3 of our series, the of LC recovery in BALF varied greatly. Possible causes of variation include: 1) washing of bronchi and alveoli that communicated with granulomas and 2) the presence of old and scarred granulomatous foci. Pulmonary granulomatosis exhibits various histological changes[11,13,18]. In patient 3, in whom a chronic scarred lesion was the main finding, the fraction rate of LCs was as high as 11%. This finding is consistent with the lungs having granulomas as well as scarring. The fraction ratio of LCs, therefore, does not directly correlate with the disease state in the present disease. Subsequently, if the present disease is suspected, performing BAL multiple times increases the probability of recovering LC and helps confirm the diagnosis.

The presence of LCs in BALF does not necessarily indicate Langerhans-cell granulomatosis[4,13,19]. According to Casolaro et al[26], LCs are always present in the lungs of healthy individuals, and recovery rates of LCs in the BALF from smokers and nonsmokers were 1.1% ± 0.03% and 0.1% ± 0.1%, respectively. According to their study using electron microscopy, the mean recovery rate of LCs in smokers was 0.4% ± 0.1%, but LCs were not seen in nonsmokers.

The number of cells recovered from BALF was usually increased, and cell counts greater than 1×10^6 cells/mL were common, with a marked predominance of alveolar macrophages. This finding reflects cigarette smoke exposure, and the macrophage count is strongly correlated with the level of daily cigarette consumption[26].

Alveolar cell counts are normal in the small minority of patients who are nonsmokers. Differential BALF cell counts may show a moderate nonspecific increase in eosinophil numbers (usually <10%)[7,27]. The percentage of alveolar lymphocytes is normal or decreased, and the CD4/CD8 ratio is decreased, as is observed in smokers.

The identification of LCs by means monoclonal antibodies directed against CD1a (OKT6 or equivalent) among cells recovered by lavage has previously been suggested to be useful as a diagnostic test in PLCH[18,28,29], but more recent evidence indicates that this approach can be disappointing and lacks sensitivity. False-positive results are common, and the specimens must be examined by an experienced cytologist. LC densities of up to 1% can be recovered by lavage from cigarette smokers without diffuse lung disease, and increased numbers of LCs (up to 3%) can be seen in patients with adenocarcinoma (bronchioloalveolar carcinoma) or diffuse interstitial lung disease associated with alveolar hyperplasia (particularly diffuse pulmonary fibrosis)[7,26]. If the threshold of 5% LCs is used for the diagnosis of PLCH in adults, our results **(Fig. 3)** and those of others[18,22,27] show that both the specificity and sensitivity are high. It remains to be established whether the clinical characteristics differ between patients with or without increased numbers of LCs on BALF.

In practice, BALF analysis rarely establishes a definite diagnosis of PLCH in adults but does provide additional orientation by showing high alveolar macrophage counts. BALF is of greatest use in the differential diagnosis of patients without typical radiological findings. The test can be used to steer the diagnosis away from interstitial lung diseases with more characteristic findings and rule out certain pulmonary infections, such as excavated forms of *Pneumocystis carinii* (recently renamed *Pneumocystis jiroveci*) pneumonia, which may cause confusion in certain cases[30].

In conclusion, if carefully checked, LCs can be thoroughly evaluated by simple observation of BALF samples stained with Giemsa. If other clinical features are present, such as 1) heavy smoking, 2) chest X-ray films characterized by distribution of micronodular shadows and cyst formation, 3) HRCT of the chest confirmed the predominant upper or mid-lung involvement, and 4) no marked clinical symptoms, except for dry cough, mild dyspnea, or pneumothorax, BALF analysis for LCs could provide valuable information in establishing the diagnosis of PLCH without the need for lung biopsy, which has an inherent risk of complications.

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