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The T cell receptor subsets of lymphocytes in bronchoalveolar lavage in patients with active pulmonary tuberculosis

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Study objective. To determine whether or not the levels of γ/δ lymphocytes increase in bronchoalveolar lavage (BAL) fluid from patients with pulmonary tuberculosis.
Design. Prospective data collection relating to cells in BAL fluid and peripheral blood mononuclear cells (PBMC) from patients with pulmonary tuberculosis and control subjects.
Setting. A university hospital, from March 1990 to December 1993.
Patients. Thirteen patients with pulmonary tuberculosis who were diagnosed by culture of *Mycobacterium tuberculosis* from their sputum of BAL fluid and/or clinical response were enrolled in the study. Fifteen healthy volunteers participated as control subjects.
Measurements and results. The differential cell counts in BAL fluid were made by Diff-Quik stain. The percentages of T-cell receptor (TCR) (γ/δ and α/β)-positive lymphocytes and interleukin 2 (IL-2) receptor-positive CD3 lymphocytes in BAL fluid and peripheral blood were measured by dual scan with flow-cytometry. The percentage and absolute number of lymphocytes and the percentages of $CD3^+$, $IL2R^+$ lymphocytes in BAL fluid significantly increased in patients with tuberculosis when compared with those of control subjects. The percentages and numbers of γ/δ and α/β TCR-positive lymphocytes in BAL fluid and PBMC from patients with tuberculosis and indistinguishable from those of control subjects.
Conclusions. γ/δ Lymphocytes do not appear to have as much meaning in patients as they do in animal studies.

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Introduction

The immunology of pulmonary tuberculosis is a complicated matter and is not clearly understood. However, T lymphocytes are involved in cell-mediated immune reactions in the pathogenesis of pulmonary tuberculosis by secreting various cytokines and chemotactic materials (1). The activation of lymphocytes may be confirmed indirectly by the elevated number of soluble interleukin 2 receptors in peripheral blood from patients with active pulmonary tuberculosis (2).

Most T lymphocytes recognize antigens through the T cell receptor (TCR), which is composed of α/β and γ/δ chains (3). The α/β receptor-bearing lymphocytes (α/β lymphocytes) have CD4 and CD8 cell-surface molecules which are involved in the major histocompatibility complex. In contrast to α/β cells, the γ/δ receptor-bearing lymphocytes (γ/δ lymphocytes) do not have CD4 and CD8 molecules. The γ/δ lymphocytes are thought to be the first defence mechanism in immunity (4). The elevation of $CD4^-$, $CD8^-$ T lymphocytes in pleural fluid from patients with tuberculous pleurisy suggested that the γ/δ lymphocytes act as defence mechanisms against *Mycobacterium tuberculosis* (*M. tuberculosis*) (5). Recently, Ito *et al.* reported that the number of γ/δ lymphocytes increased in peripheral blood from patients with active pulmonary tuberculosis (6). However, the target organ of pulmonary tuberculosis is not peripheral blood but the lungs. The lymphoblast transformation to purified protein derivatives was depressed in peripheral blood, but higher in pleural fluid from patients with tuberculous pleurisy, which was explained by the phenomenon of sequestration (7). To the present authors' knowledge, no one has yet measured the level of γ/δ lymphocytes in BAL fluid from patients with pulmonary tuberculosis. The previous data suggest that it is important to measure the level of γ/δ lymphocytes in bronchoalveolar lavage (BAL) fluid, which mirrors the cell

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composition of target organs in patients with pulmonary tuberculosis.

The present study investigated the level of γ/δ lymphocytes in BAL fluid from patients with active pulmonary tuberculosis, comparing that level with those in peripheral blood and that in BAL fluid from normal subjects.

Materials and Methods

PATIENTS

Thirteen patients with active pulmonary tuberculosis, four females and nine males, were included in this study after informed consent was obtained. Their ages ranged from 23 to 60 years. Of these 13, 11 were later diagnosed by *M. tuberculosis* culture and two by positive smear of acid-fast bacilli (AFB) with good clinical and radiological response to antituberculous chemotherapy. They denied having had any previous history of pulmonary tuberculosis and chemotherapy.

Fifteen healthy non-smoking volunteers consisting of 14 males and one female were also included in this study after giving informed consent. They had no respiratory symptoms for at least 1 month prior to the study, and their chest radiographs were normal. Their ages ranged from 27 to 31 years.

METHODS

The isolation of peripheral blood mononuclear cells (PBMC) was performed on the day of bronchoscopy by the density gradient method as previously described (8). Briefly, 10 ml heparinized peripheral blood, diluted with phosphate-buffered salts (PBS) solution, was centrifuged on the Ficoll-hypaque solution. The PBMC were washed twice with Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (HBSS) and suspended in HBSS containing 0·1% bovine serum albumin and 0·2% sodium azide. Flow-cytometric analysis was performed by the description below.

Bronchoalveolar lavage was carried out by one operator as previously described (9). The premedication was with diazepam and atropine. The procedure was performed by instilling 50 ml normal saline four times in the most affected lung, which was determined by chest X-ray. Negative pressure below 50 mmHg was applied for suction under fiberoptic bronchoscopy (Olympus B2-10; Olympus Optical Co., Tokyo, Japan). Cell pellets and supernatants were separated by centrifugation at 500 ***g*** for 5 min. A differential cell count up to 500 cells was done on slides prepared by cytocentrifuge and Diff-Quik stain (Scientific products, Gibbstowne, USA).

BAL cells were washed twice with Ca^{2+}- and Mg^{2+}-free HBSS and suspended in HBSS containing 0·1% bovine serum albumin and 0·2% sodium azide. Specific binding of monoclonal antibodies (mAbs) was analysed by dual immunofluorescence staining according to standard methods recommended by Becton-Dickinson Monoclonal Center (Mountain View, USA). Aliquots containing 1×10^6 cells were incubated for 30 min at 4°C in the presence of saturating concentrations of fluorescein isothiocyanate (FITC) and phycoerythrin-conjugated mAbs. mAbs for CD3 (anti-Leu-4) in conjugated form with FITC and CD25 (anti-Tac), anti-TCR-γ1, anti-TCR1 in conjugated form with phycoerythrin were obtained from Becton Dickinson. The cells were washed in PBS solution and fixed in 1 ml of 1% paraformaldehyde in PBS solution.

Cytofluorometric analysis of the cells for the surface immunofluorescence was performed with FACScan (Becton-Dickinson). The cells were excited with a 488 nm argon laser. The filter set used to separate the two signals was recommended by the manufacturer for FITC and phycoerythrin. For each sample 1×10^4 cells were analysed. The data were collected and analysed on the Consort 40 program (Becton-Dickinson).

STATISTICAL ANALYSIS

The comparisons between the cellular compositions of the patients with pulmonary tuberculosis and the control group and between BAL fluid and PBMC were made by the Mann–Whitney *U* test and Wilcoxon rank sum test, respectively. All data are expressed as mean ± SEM with *P* values <0·05 being considered statistically significant.

Results

The detailed profiles of BAL fluid from the patients and healthy studied subjects are shown in Table 1 and Table 2. The lavage site was the right upper lobe in nine cases, right lower lobe in three cases, and right middle lobe in one case. The recovered lavage volume was not different between patients with tuberculosis and control subjects (102 ± 3·7 *vs.* 110 ± 6·6 ml, $P>0·05$). However, total cell numbers in 1 ml of BAL fluid and the percentage and absolute number of lymphocytes in 1 ml of BAL fluid significantly increased in patients with tuberculosis when compared with those of control subjects. The percentage of macrophages was decreased in patients with tuberculosis when compared with those of control subjects although the absolute numbers were increased. The composition of neutrophils did not show any difference between in-patients with tuberculosis and control subjects (Table 3).

The percentages of $CD3^+$ lymphocytes in BAL fluid from patients with tuberculosis and control subjects were 74·9 ± 2·6% and 85·4 ± 7·5%, which were higher ($P<0·05$) than those in PBMC (58·6 ± 2·8% and 59·4 ± 4·1%, respectively). The percentage of $CD3^+$ lymphocytes in BAL fluid was higher in patients with tuberculosis than it was in control subjects in BAL fluid (Fig. 1). The percentages of $CD3^+$, $IL2R^+$ lymphocytes in either BAL fluid or PBMC were higher in patients with tuberculosis than they were in control subjects. However, the percentages were not different between BAL fluid (12·65 ± 7·01%) and PBMC (3·34 ± 1·19%) (Fig. 2).

The percentages of γ/δ lymphocytes in BAL fluid and PBMC from patients with tuberculosis were 3·23 ± 0·78% and 7·61 ± 2·06%, which was indistinguishable from control subjects (6·9 ± 0·93% and 9·52 ± 1·72%, respectively).

TABLE 1. Clinical and BAL findings in patients with pulmonary tuberculosis

Number	Age	Sex	Smoking (pack-years)	Diagnosis (AFB study)	Lavage site	Recovered volume (ml)	Total cell count ($\times 10^4$ ml^{-1})	Macrophage ($\times 10^4$ ml^{-1}, %)	Lymphocyte ($\times 10^4$ ml^{-1}, %)	Neutrophil ($\times 10^4$ ml^{-1}, %)
1	23	F	Non-smoker	Smear$^-$ Culture$^+$	RUL	80	56	52·4 (94)	2·3 (4)	1·7 (3·0)
2	45	F	Non-smoker	Smear$^-$ Culture$^+$	RUL	90	84	29·9 (36)	53·6 (64)	0·5 (0·6)
3	30	F	Non-smoker	Smear$^+$ Culture$^+$	RLL	96	45	28·0 (62)	15·3 (34)	1·2 (2·6)
4	52	F	Non-smoker	Smear$^-$ Culture$^+$	RUL	·98	18	3·6 (20)	14·3 (79)	0·1 (0·4)
5	59	M	Smoker (20)	Smear$^-$ Culture$^+$	RUL	100	33	9·9 (30)	21·3 (64)	0·8 (2·5)
6	24	M	Smoker (2)	Smear$^-$ Culture$^+$	RLL	102	16	10·1 (63)	5·6 (35)	0·1 (0·6)
7	27	M	Non-smoker	Smear$^-$ Culture$^-$	RUL	102	32	20·2 (63)	12·2 (38)	0·7 (2·2)
8	43	M	Ex-smoker (10)	Smear$^-$ Culture$^-$	RLL	110	43	23·9 (56)	18·5 (43)	0·5 (1·2)
9	60	M	Non-smoker	Smear$^+$ Culture$^+$	RML	130	26	16·4 (63)	8·2 (32)	0·6 (2·2)
10	58	M	Non-smoker	Smear$^-$ Culture$^+$	RUL	114	46	24·7 (54)	17·7 (38)	2·1 (4·6)
11	54	M	Non-smoker	Smear$^+$ Culture$^+$	RUL	98	45	24·4 (54)	15·9 (35)	3·9 (8·6)
12	56	M	Ex-smoker (10)	Smear$^+$ Culture$^+$	RUL	90	14	9·4 (67)	3·9 (28)	0·5 (3·4)
13	49	M	Non-smoker	Smear$^-$ Culture$^+$	RUL	124	49	40·7 (83)	6·4 (13)	0·3 (0·7)

RUL, right upper lobe; RLL, right lower lobe; RML, right middle lobe.

TABLE 2. BAL findings in healthy subjects*

Number	Age	Sex	Recovered volume (ml)	Total cell count ($\times 10^4$ ml^{-1})	Macrophage ($\times 10^4$ ml^{-1}, %)	Lymphocyte ($\times 10^4$ ml^{-1}, %)	Neutrophil ($\times 10^4$ ml^{-1}, %)
1	27	M	100	12	9·0 (75)	2·9 (23·9)	0·1 (1·1)
2	31	M	95	21	18·6 (89)	2·3 (11·0)	0·1 (0·3)
3	28	M	94	13	11·1 (85)	1·8 (13·7)	0·2 (1·2)
4	28	F	160	9	8·3 (92)	0·3 (3·8)	0·3 (3·5)
5	29	M	104	16	14·2 (89)	1·6 (10·0)	0·2 (1·4)
6	30	M	85	20	17·4 (87)	2·0 (9·8)	0·7 (3·4)
7	31	M	120	23	21·7 (94)	0·3 (1·5)	0·9 (4·0)
8	27	M	105	23	21·6 (94)	0·9 (3·9)	0·5 (2·1)
9	27	M	120	16	11·3 (70)	4·6 (28·9)	0·1 (0·5)
10	28	M	136	21	19·7 (94)	0·8 (3·7)	0·5 (2·5)
11	29	M	130	4	3·6 (90)	0·4 (8·9)	0·1 (1·3)
12	31	M	85	6	5·6 (93)	0·3 (4·6)	0·1 (2·4)
13	29	M	140	8	7·7 (97)	0·2 (2·1)	0·1 (1·2)
14	31	M	56	45	42·5 (95)	1·8 (4·0)	0·7 (1·5)
15	29	M	125	16	15·1 (94)	0·3 (1·8)	0·6 (3·9)

*All subjects are non-smokers. Instilled volume is 200 ml and the lavage site is right middle lobe in all cases.

TABLE 3. The summary of cellular profiles in patients with tuberculosis and healthy subjects

	Healthy subjects	Tuberculosis	*P* value
Total cell count ($\times 10^4$ ml^{-1})	16·9 ± 2·5	39·0 ± 5·1	<0·05
Macrophages			
Number ($\times 10^4$ ml^{-1})	15·2 ± 2·3	22·6 ± 3·6	<0·05
Percentage	89·2 ± 1·9	57·3 ± 5·4	<0·05
Lymphocytes			
Number ($\times 10^4$ ml^{-1})	1·4 ± 0·3	15·0 ± 3·5	<0·05
Percentage	8·8 ± 2·0	39·2 ± 5·5	<0·05
Neutrophils			
Number ($\times 10^4$ ml^{-1})	2·0 ± 0·3	2·5 ± 0·6	n.s.
Percentage	0·3 ± 0·1	1·0 ± 0·3	n.s.

n.s.=not significant.

Differences were not seen when comparing the percentages in BAL fluid and peripheral blood in patients with tuberculosis and control subjects (Fig. 3). The absolute number of γ/δ lymphocytes per millilitre, which was obtained from the percentage of lymphocytes in BAL fluid multiplied by the cell number per millilitre of BAL fluid increased significantly in patients with tuberculosis (3554 ± 765 ml^{-1}) when compared with that of control subjects (874 ± 240 ml^{-1}) (Fig. 4).

There were no significant differences between patients with pulmonary tuberculosis and healthy subjects in the percentages of α/β lymphocytes in BAL fluid and those in PBMC. However, the absolute count of α/β lymphocytes in BAL fluid was significantly higher in patients with pulmonary tuberculosis than in healthy subjects (Fig. 4). The ratio of composition of $\alpha\beta/\gamma\delta$ lymphocytes was significantly higher in patients with pulmonary tuberculosis than in healthy subjects (Fig. 5).

Discussion

This study showed that the percentage of γ/δ lymphocytes in BAL fluid from patients with active pulmonary tuberculosis was not different from that of healthy volunteers. This finding is in distinction to those showing increased numbers of activated CD3$^+$, α/β-TCR$^-$ (suggesting γ/δ lymphocyte) after exposure of aerosols containing *M. tuberculosis* in mice (10) and increased expression of γ/δ TCR after co-culture with *M. tuberculosis* and lymphocytes in patients with protective immunity (tuberculin reactors and tuberculous pleuritis) (11). This discrepancy may be related to the interval between the exposure of *M. tuberculosis* and the time of study. In animal studies (10,11), the exact time the animals were exposed to *M. tuberculosis* could be determined; however, in this study, determining the exact time of exposure to *M. tuberculosis* was not possible. Inoue *et al.* (12) reported that peritoneal exudate cells expressing

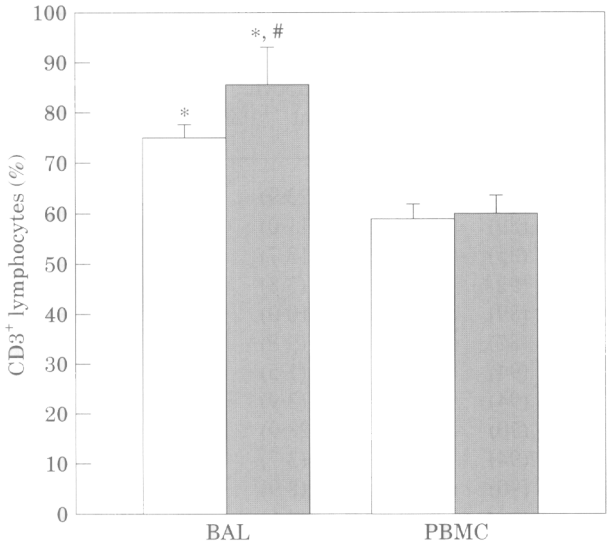

FIG. 1. The percentages of $CD3^+$ lymphocytes in BAL fluid and PBMC from control subjects (open bar) and patients with tuberculosis (hatched bar). $^*P<0{\cdot}05$ *vs.* PBMC; $^\#P<0{\cdot}05$ *vs.* the control subjects.

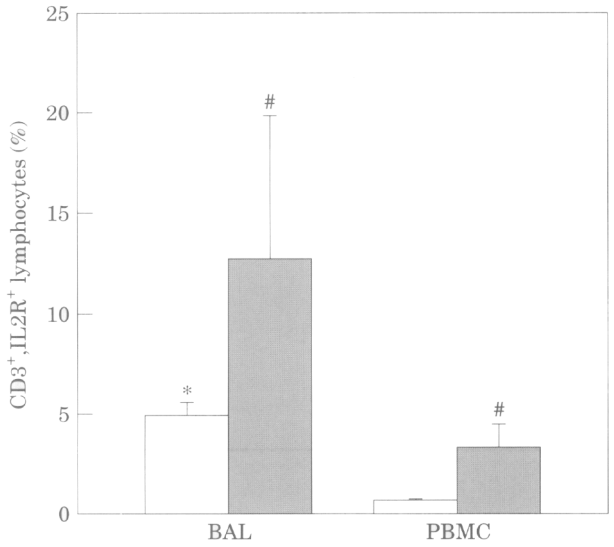

FIG. 2. The percentages of $CD3^+$, $IL2R^+$ lymphocytes in BAL fluid and PBMC from control subjects and patients with tuberculosis. The symbols are the same as those in Fig. 1.

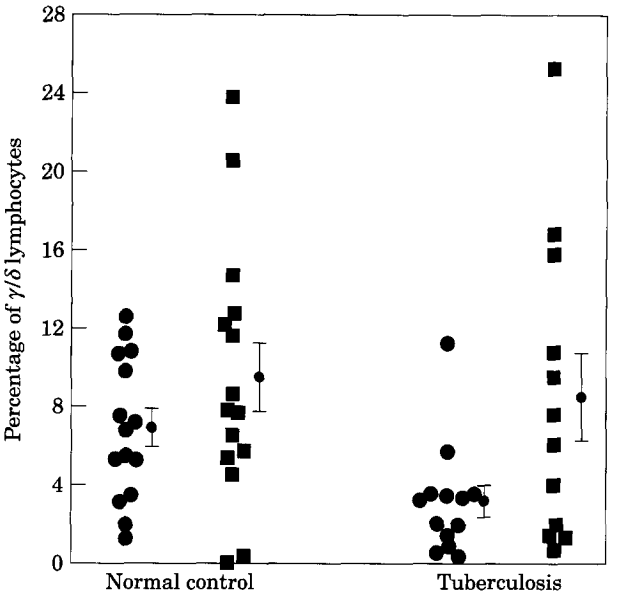

FIG. 3. The percentages of γ/δ lymphocytes in BAL fluid (solid circle) and PBMC (solid square) from patients with tuberculosis and control subjects.

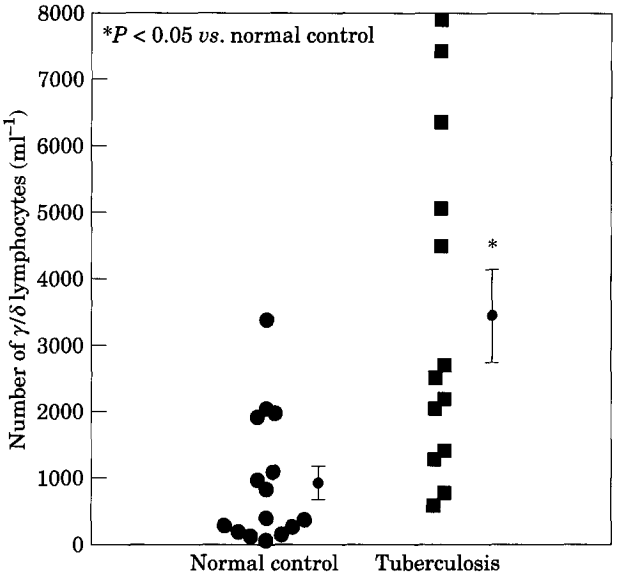

FIG. 4. The absolute number of γ/δ lymphocytes in BAL fluid from patients with tuberculosis and control subjects. The asterisk denotes $P<0{\cdot}05$ *vs.* control subjects.

γ/δ TCR decreased gradually, from 7 to 28 days, after exposure of BCG. In addition, Ohga *et al.* (13) described the sequential appearance of γ/δ and α/β-bearing T-cells during infection with *Listeria monocytogenes*. They observed the peak level of γ/γ chain gene on day 3 and of α/β chain gene on day 8 after exposure to *L. monocytogenes*. By these findings, they suggested that γ/δ T-cells precede the α/β T-cells in appearance during *Listeria* infection. Although *M. tuberculosis* differs from *L. monocytogenes* in terms of antigen, γ/δ lymphocytes act as a primary immune response to certain micro-organisms (14). This sequential appearance of α/β and γ/δ lymphocytes may explain our result. Furthermore, the vast majority of infiltrating lymphocytes were α/β rather than γ/δ lymphocytes in granulomata from patients with tuberculous lymphadenitis (15). Tazi *et al.* (16) observed that the proportion and absolute number of circulating γ/δ lymphocytes are not different when comparing blood from patients with tuberculosis and control subjects. These results support our findings. Therefore, it is possible to say that, in instances when the exact time of exposure to antigen cannot be identified, such as this clinical study, γ/δ lymphocytes do not increase because of

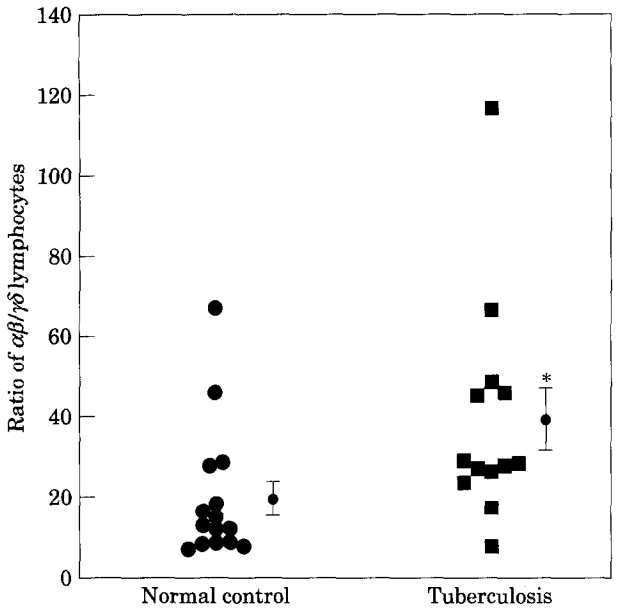

FIG. 5. The ratio of $\alpha\beta/\gamma\delta$ lymphocytes in BAL fluid from patients with tuberculosis and control subjects. $^*P<0{\cdot}05$ *vs.* control subjects.

the switching of γ/δ lymphocytes to α/β lymphocytes. The other possible causes of the discrepancy might be species differences and kinetic factors in terms of primary immunization and secondary reaction (16). In addition, it is difficult to say that the γ/δ lymphocytes have important roles in the pathogenesis of pulmonary tuberculosis by the finding of elevated absolute numbers of γ/δ lymphocytes in BAL fluid from patients with pulmonary tuberculosis, because patients with tuberculosis had an elevated level of the ratio of $\alpha\beta/\gamma\delta$ lymphocytes and absolute number of $\alpha\beta$ lymphocytes. These findings suggest that they may be switching from $\gamma\delta$ to $\alpha\beta$ lymphocytes *in vivo*.

It is very important to compare the cellular composition between the radiographically normal and abnormal sites in the same individual for understanding the local cellular response to *M. tuberculosis*. However, in this study, it was impossible to perform the BAL at diseased and healthy sites because of ethical problems and difficulty in getting informed consent from patients and volunteers. Thus, further researches should include the BAL at the normal and abnormal sites for the evaluation of cellular response to *M. tuberculosis*.

We found the composition of IL-2R-expressing lymphocytes in BAL fluid was higher than that in PBMC in healthy subjects, suggesting that the cells are activated in normal lung. This finding is consistent with the high proportion of HLA-DR-positive T cells in normal lavage fluid (17) and higher expression of IL-2R and HLA-DR in $CD4^+$ and $CD8^+$ BAL T cells than in blood T cells (18). Although the 'activation' of lymphocytes in BAL fluid in control subjects might reduce the significance of the 'activation' of cells in patients with pulmonary tuberculosis, we neglected the activation of lymphocytes in control subjects because the proportion of IL-2R-expressing lymphocytes in healthy subjects was lower than that in patients with pulmonary tuberculosis.

Smoking induced alveolar cellular responses resulting in the elevation of the number of alveolar cells, predominantly macrophages. In contrast to macrophages, the absolute number and subtypes of lymphocytes was not statistically different between non-smokers and smokers (19). These findings may suggest that smoking itself did not affect the analysis of results in this study because we have focused on the lymphocytes.

If diseases are localized, as evidenced by focal infiltrates in patients with interstitial lung diseases, the cellular profiles of lavage fluid can show different results (20). By these findings, in this study, the bronchoalveolar lavage had to be performed at the most affected lesion, mainly the right upper lobe, which is the predisposing target of pulmonary tuberculosis. The BAL cellular composition of neutrophils and eosinophils was not dependent on the lavage site in normal subjects (21), and the differences were not observed in the cell components of macrophages, neutrophils and lymphocytes in patients with pulmonary sarcoidosis (22). Thus, we performed the lavage in the right middle lobe instead of the right upper lobe in normal controls because of easy access and no interlobar cellular differences.

In summary, we have shown that lavaged alveolar lymphocytes express higher IL-2R levels, but do not show higher levels of γ/δ lymphocytes in patients with pulmonary tuberculosis than in control subjects. γ/δ lymphocytes do not appear to have as much meaning in patients as in the animal studies.

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