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USEFULNESS OF *MYCOBACTERIUM TUBERCULOSIS*-SPECIFIC ENZYME-LINKED IMMUNOSPOT ASSAY FOR THE DIAGNOSIS OF TUBERCULOUS PLEURISY

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SUMMARY

The diagnosis of tuberculous pleurisy by the analysis of pleural fluid (PF) is difficult using standard diagnostic methods. The present study examined the usefulness of *Mycobacterium tuberculosis* (MTB)-specific enzyme-linked immunospot (ELISpot) assay for the diagnosis of tuberculous pleurisy in Japanese patients. Both peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) were obtained from 6 patients with tuberculous pleurisy and 10 patients with non-tuberculous pleurisy. We analyzed 2×10^5 cells/well of MTB-specific ELISpot assay stimulated by early secretory antigenic target-6 (ESAT-6) antigen and phytohemagglutinin (PHA). Numbers of Spot-forming cells (SFCs) treated by ESAT-6 in PBMCs did not differ significantly between tuberculous and non-tuberculous pleurisy patients. In contrast, numbers of SFCs treated by ESAT-6 were sig-

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Abbreviations: PF, pleural fluid; MTB, *Mycobacterium tuberculosis*; ELISpot, enzyme-linked immunospot; ESAT-6, early secretary antigenic target-6; ELISA, enzyme-linked immunosorbent assay; PBMCs, peripheral blood mononuclear cells; PFMCs, pleural fluid mononuclear cells; SFCs, spot-forming cells; PHA, phytohemagglutinin; E/P ratio, ratio of ESAT-6 SFCs to PHA SFCs; ADA, adenosine deaminase; BCG, Bacille Calmette Guerin; RD-1, region of difference1; AFB, acid fast bacilli; TST, tuberculin skin test; WBC, white blood cell; N, neutrophil; L, lymphocyte; yrs, years; CRP, C-reactive protein; LTBI, latent tuberculous infection; IFN, interferon;
DNA, deoxyribonucleic acid

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nificantly higher in PFMCs in tuberculous pleurisy patients than in non-tuberculous pleurisy patients (p<0.01). Furthermore, the ratio of ESAT-6 SFCs to PHA SFCs (E/P ratio) among PFMCs was significantly higher for tuberculous pleurisy than for non-tuberculous pleurisy. These results indicate that MTB-specific ELISpot assay is useful for rapid diagnosis of tuberculous pleurisy.

Key words: tuberculous pleurisy; ELISpot; PHA; E/P ratio

INTRODUCTION

Tuberculosis is one of the most important infectious causes of mortality and morbidity worldwide, with approximately 2 million deaths annually and 9 million new patients reported in 2004 (1). In Japan, about 26,000 new cases of tuberculosis and 2000 deaths occur annually (2). Among tuberculous patients in Japan, around 5000 patients develop complicated pleurisy (3). Tuberculosis-related pleurisy is seen in about 25% of patients with various diseases displaying pleural fluid (PF) (4). Although microbiological and histological examination is needed to diagnose tuberculous pleurisy, the sensitivity of microbiological examination is not high, and invasive histological examination sometimes causes complications (5-8). In many cases, tuberculous pleurisy is clinically diagnosed by adenosine deaminase (ADA) levels and differential lymphocyte counts in PF. Although ADA offers a useful parameter in terms of sensitivity and specificity, some patients cannot be diagnosed using this test alone (9). We have therefore been searching for new methods to directly diagnose tuberculous pleurisy with PF.

Mycobacterium tuberculosis (MTB)-specific T cell interferon (IFN)-γ-releasing assays by enzyme-linked immunospot (ELISpot) or enzyme-linked immunosorbent assay (ELISA) have recently offered the promise of diagnosing MTB infection from peripheral blood (10-12). These assays use peptides from early secretary antigenic target-6 (ESAT-6), which is encoded by region of difference 1 (RD-1) in the MTB genome, a region that is absent from all strains of *Mycobacterium bovis* Bacille Calmette Guerin (BCG) vaccine and most non-tuberculous mycobacteria (13, 14). These assays are thus unaffected by prior BCG vaccination.

The present study examined the usefulness of the ELISpot assay stimulated by ESAT-6 antigen for the diagnosis of tuberculous pleurisy in Japanese patients.

MATERIALS AND METHODS

Patients

Both peripheral blood mononuclear cells (PBMCs) and pleural fluid mononuclear cells (PFMCs) were obtained from 16 patients with PF who had been admitted to Nagoya City University Hospital or Kasugai Municipal Hospital. All study protocols were approved by the local ethics com-

mittee and informed consent had been obtained from all patients. PBMCs were obtained from blood sample (26 ml) and PFMCs were obtained from PF (100 ml). For the diagnosis of tuberculosis, we examined bacterial culture and MTB-specific deoxyribonucleic acid (DNA) amplification for each patient. Tuberculous pleurisy was confirmed in 6 patients (6 men) with a median age of 80. 5 years. Lateral PF with origins other than tuberculosis was identified in 10 patients (6 men, 4 women) with a median age of 79 years, and these were included in the study as controls.

Definition of cases and controls

Patients with tuberculosis were defined as follows: positive results for microbiological examination or MTB-specific DNA amplification from sputum or pleural effusion. Patients diagnosed with non-tuberculous pleurisy were defined as control cases.

ELISpot assay

ELISpot assay for human IFN-γ was performed as described elsewhere within 12 h after obtaining blood (16 ml) and PF (50 ml)(15). PBMCs were prepared by centrifugation from heparinized blood with a cell preparation tube (BD Vacutainer CPT; Becton, Deckinson and Company, United states). PFMCs were prepared by Ficoll-Hypaque gradient centrifugation from PF. PBMCs (2×10^5 cells/well) and PFMCs (2×10^5 cells/well) were plated overnight on 96-well plates (MILLIPORE Multiscreen; Millipore Corporation, France) that had been pre-coated with anti-human IFN-γ antibody, in 200 μl of culture medium per well. Cells were left unstimulated, or were stimulated with phytohemagglutinin (PHA) (25 μg/ml; Wako, Japan), with ESAT-6 (25 μg/ml recombinant dual ESAT-6; Statens Serum Institut, Denmark).

Culture of the plates, washing, counterstaining and visualization were performed according to the guidelines supplied by the manufacturer. Negative control well spots were subtracted from the number of spots counted in wells stimulated by ESAT-6. Counts of spot numbers were determined by two persons blinded to clinical characteristics and outcomes, and then mean data were used for analysis.

Statistical analysis

All statistical tests were performed as exploratory analysis without adjustment for multiple testing, with normal significance defined as $p<0.01$. Continuous variables were compared by nonparametric testing (Mann-Whitney test), as the data were not normally distributed.

RESULTS

In 6 tuberculous pleurisy patients, 3 patients were diagnosed by PF samples, 2 patients were diagnosed by sputum samples and 1 patient was diagnosed by both sputum and PF samples. In the 10 non-

tuberculous pleurisy patients, 7 patients had malignant PF (non-small cell lung cancer, n = 6; renal cell cancer, n = 1), 1 patient had empyema and 2 patients had exudative pleurisy (Table 1).

ESAT-6 specific cells in PFMC samples were more highly concentrated in patients with tuberculous pleurisy than controls ($p < 0.01$), and no significant differences were observed in PBMC samples (Fig. 1A). The numbers of PHA spot-forming cells (SFCs) in PF or peripheral blood were not significantly different between controls and patients with tuberculous pleurisy (Fig. 1B). The ratios of ESAT-6 SFCs to PHA SFCs (E/P ratios) in PFMC samples were significantly higher in patients

TABLE 1. Characteristics of TB and non-TB patients

Case	Age (yrs)	Sex	Diagnosis	WBC (N,L)	CRP	AFB	MTB culture	MTB NAT
1	59	M	TB pl	4.9 (70,16)	1.0	-	-	+ (P)
2	87	M	TB pl	4.0 (51,26)	1.0	-	+ (P)	+ (P)
3	82	M	TB pl + pTB	6.0 (73,13)	8.7	+ (S)	+ (S, P)	+ (S)
4	63	M	TB pl + pTB	10.3 (68,20)	5.1	-	+ (S)	-
5	86	M	TB pl + pTB	9.0 (75,11)	10.5	-	+ (S)	+ (S)
6	79	M	TB pl + mTB	10.9 (74,18)	5.2	-	+ (P)	-
7	75	F	Lung ca	5.9 (61, 32)	0.7	-	-	-
8	77	M	Lung ca	6.0 (79, 10)	8.6	-	-	-
9	73	F	Renal ca	8.6 (80, 10)	2.4	-	-	-
10	84	M	Ex pl	6.5 (72, 15)	2.0	-	-	-
11	80	M	Lung ca	8.3 (77, 15)	0.4	-	-	-
12	94	M	Empyema	18.7 (95, 2)	18.0	-	-	-
13	68	M	Lung ca	6.7 (70, 20)	0.1	-	-	-
14	84	F	Ex pl	6.3 (90, 8)	4.6	-	-	-
15	64	F	Lung ca	8.5 (61, 32)	5.6	-	-	-
16	65	M	Lung ca	8.4 (64, 19)	6.2	-	-	-

M, male; F, female; yrs, years; TB pl, tuberculous pleurisy; pTB, pulmonary tuberculosis;
mTB, milliary tuberculosis; Ex pl, exudative pleurisy; ca, carcinoma; WBC ($10^3/\mu l$), white blood cell;
N (%), neutrophil; L (%), lymphocyte; CRP (mg/dl), C-reactive protein; AFB, acid-fast bacilli;
S, sputum; P, pleural fluid; NAT, nucleic acid amplification technique;

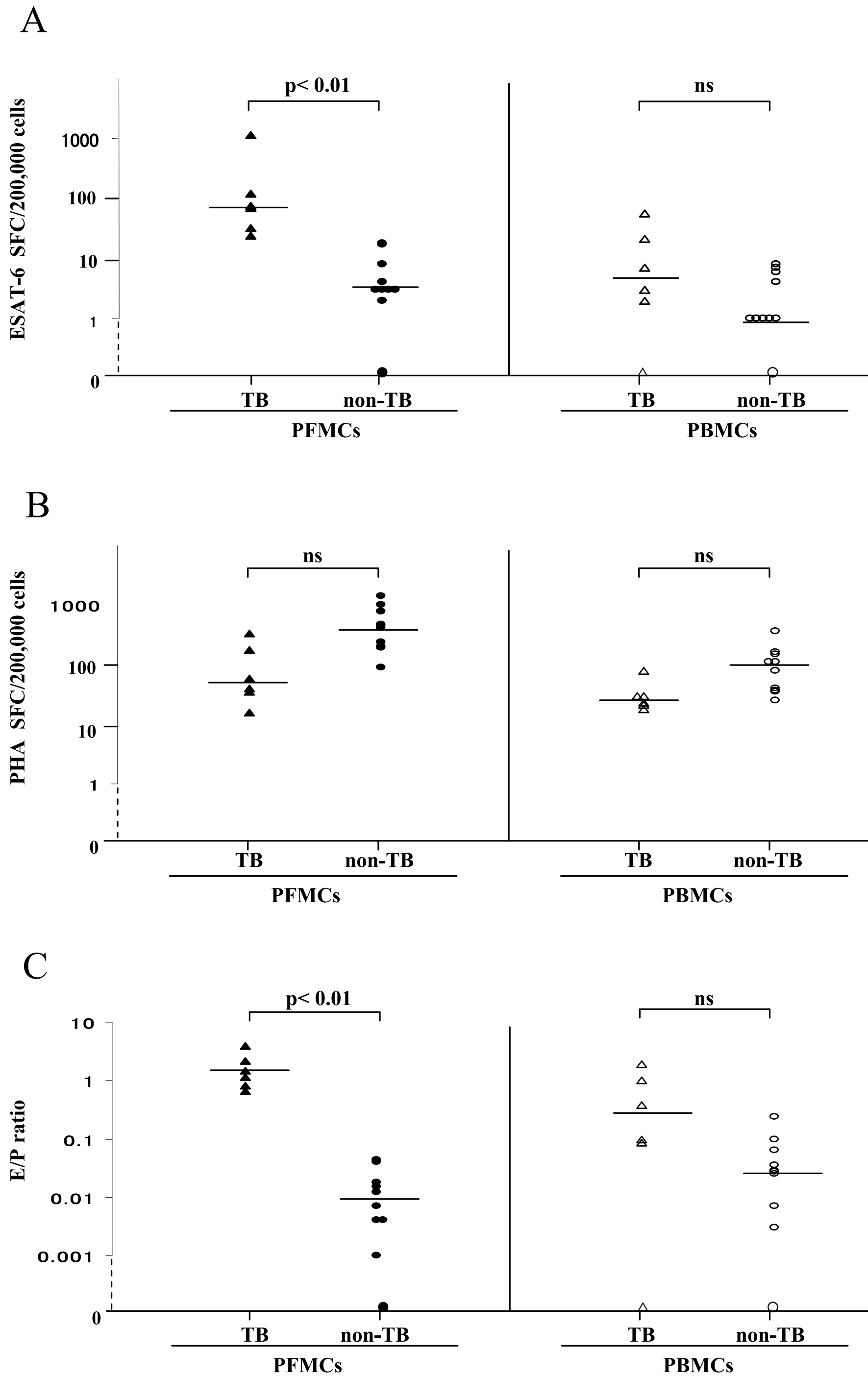

FIG. 1. Numbers of ESAT-6, PHA SFCs and E/P ratio in PBMCs or PFMCs of patients with TB (n = 6, PFMCs; ▲ and PBMCs; △) and non-TB. (n = 10, PFMCs; ● and PBMCs; ○). A, ESAT-6 SFC; B, PHA SFC; C, E/P ratio. All results have been calculated after subtraction of SFCs in negative control wells. Bars represent median values. ns; not significant.

with tuberculous pleurisy than those with non-tuberculous pleurisy (p<0.01), and no significant differences were observed in PBMC samples (Fig. 1C). Although ESAT-6 SFCs in PFMCs in Case 6 of the tuberculous pleurisy group, resembled those in Cases 7 and 8 of non-tuberculous pleurisy (24 vs. 18, 17), E/P ratio in Case 6 was significantly higher than in Cases 7 or 8 (1.5 vs. 0.018, 0.012).

DISCUSSION

The gold standard for the diagnosis of tuberculous pleurisy is culture of MTB, detection of MTB-DNA, detection of alcohol-acid fast bacilli (AFB), and histopathological detection of caseating granulomas by pleural biopsy. Culture of MTB, detection of MTB-DNA, and detection of AFB from pleural fluids do not provide high sensitivity. These tests show a sensitivity for tuberculous pleurisy ranging from 12-70% (with the majority of studies showing sensitivity < 30%), 30-100% (in culture-negative cases, 30-60%), and < 10%, respectively (3, 7, 8).

Antigen-specific IFN-γ-releasing T cells are key components of the host response to the MTB (16). MTB-specific IFN-γ-release assays have been developed as ELISpot and ELISA for the diagnosis of MTB infection from peripheral blood (10-12). These assays use the ESAT-6 antigen, encoded in the genomic region of difference 1 (RD-1) of MTB, which is absent in most non-tuberculous mycobacteria, including vaccination strains of *Mycobacterium bovis* BCG (10-12). In contrast to the tuberculin skin test (TST), MTB-specific ELISpot is not influenced by prior BCG vaccination and has lower cross-reactivity to mycobacteria other than MTB. However, like the TST, MTB-specific ELISpot does not allow distinction between active and latent tuberculous infection (LTBI) when performed on blood (17). Recently, Kang, et al reported that the specificity of ELISpot assay on blood was low for active tuberculosis (47%), although the sensitivity was high (92%) (18). In our study, MTB-specific ELISpot on blood did not differ significantly between tuberculous pleurisy and non-tuberculous pleurisy patients.

In tuberculous pleurisy, concentrations of T lymphocytes are significantly higher in PF than in peripheral blood, while this difference is not observed in patients with non-tuberculous pleurisy. One study reported that ESAT-6-specific IFN-γ-releasing T cells are concentrated a mean of 15-fold in tuberculous PF, relative to levels in peripheral blood (19). Enumerating ESAT-6-specific IFN-γ-releasing T cells by ELISpot on PF should thus enable more specific diagnosis of tuberculous pleurisy than enumerating IFN-γ-releasing T cells in blood.

In a study of the diagnosis of tuberculous pleurisy, MTB-specific ELISpot in a routine clinical practice setting by counting antigen-specific mononuclear cells from peripheral blood and PF of patients with exudative pleurisy offered a diagnostic sensitivity for tuberculous pleurisy of 95%, but the diagnostic specificity of MTB-specific ELISpot was 76%, lower than that of ADA in PF (20). We analyzed MTB-specific ELISpot assay stimulated with ESAT-6 and E/P ratio in PFMCs differed sig-

nificantly between tuberculous pleurisy and control patients.

The quality of a PBMCs and PFMCs sample affects response detection in the IFN-γ ELISpot assay. Multiple factors can influence the quality of PBMCs and PFMCs preparations from the time of blood and PF collection through the point of sample testing (21). Therefore, the SFCs had better to be corrected, though ELISpot assay is standardized by the absolute numbers of antigen-specific IFN-γ-releasing T cells. In fact our data show that in Case 6 of the tuberculous pleurisy group, absolute numbers of ESAT-6 SFCs in PFMCs resembled those in Cases 7 and 8 of non-tuberculous pleurisy (24 vs. 18, 17), but E/P ratio was significantly higher in Case 6 than in Cases 7 or 8 (1.5 vs. 0.018, 0.012). While PHA is the mitogen to non-specifically stimulate T cells, SFCs for PHA is considered a surrogate marker for interferon-γ-releasing T-cell counts in PF (22). We conclude that the E/P ratio of PF with ELISpot assay may be one of the most reliable markers for the diagnosis of tuberculous pleurisy.

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