Gamma Interferon Immunospot Assay of Pleural Effusion Mononuclear Cells for Diagnosis of Tuberculous Pleurisy

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Diagnosis of tuberculous pleurisy remains a challenge in the clinic. In this study, we evaluated the usefulness of a previously developed Mycobacterium tuberculosis antigen–specific gamma interferon enzyme-linked immunospot (ELISPOT) assay in the diagnosis of tuberculous pleurisy by testing a cohort of 352 patients with pleural effusion. We found that M. tuberculosis antigen–specific gamma interferon–producing cells were enriched four to five times in pleural fluid compared with their levels in peripheral blood from patients with tuberculous pleurisy assayed in parallel. The sensitivity, specificity, positive predictive value, and negative predictive value of the pleural fluid mononuclear cell ELISPOT assay for the diagnosis of tuberculous pleurisy were 95.7%, 100%, 100%, and 81.0%, respectively. In comparison, the sensitivity and specificity of the ELISPOT assay using peripheral blood mononuclear cells were 78.3% and 86.3%, respectively. The sensitivity and specificity of the pleural fluid adenosine deaminase activity test were 55.5% and 86.3%, respectively. These results demonstrate that the M. tuberculosis antigen–specific ELISPOT assay performed on pleural fluid mononuclear cells provides an accurate, rapid diagnosis of tuberculous pleurisy.

Tuberculosis (TB) is a leading cause of morbidity and mortality throughout the world, with 95% of cases and 97% of all deaths occurring in high-prevalence countries such as China, where the prevalence of active TB is as high as 367/100,000 population (1). Tuberculous pleurisy (TBP) is a common clinical manifestation of active TB disease and may account for up to 50% of all pleural effusions in areas with a high incidence of TB (2). Standard diagnostic tests for TBP, including microbiological examination, adenosine deaminase (ADA) levels, cell infiltrate profile, and certain other biochemical tests, do not provide satisfactory sensitivity and specificity (3). As an example, ADA determination has been recognized as a relatively sensitive diagnostic test for TBP; increased ADA levels are observed in a number of other diseases, such as parapneumonic effusions and noninfectious inflammatory diseases (4). High pleural fluid ADA levels do not necessarily indicate the presence of TBP, especially when pleural fluid potassium levels exceed 5.0 mEq/liter (5).

Infection with Mycobacterium tuberculosis elicits a strong antigen–specific gamma interferon (IFN-γ) response from host T cells, which is used as an indicator to diagnose latent M. tuberculosis infection in clinical practice (6, 7). In addition, we and others have applied the M. tuberculosis antigen–specific IFN-γ enzyme–linked immunosorbent spot (ELISPOT) assay to peripheral blood mononuclear cells (PBMC) to diagnose active TB (8, 9). Although the sensitivity of the peripheral blood ELISPOT assay for the diagnosis of active pulmonary TB is significantly higher than those of routine tests such as sputum M. tuberculosis culture, the specificity is not satisfactory, mostly due to its inability to discriminate active TB disease from latent M. tuberculosis infection (8, 9). Previous studies have demonstrated that M. tuberculosis antigen–specific CD4+ T cells are recruited to the site of infection and that pleural fluid is enriched with antigen–experienced T cells compared with matched peripheral blood (10). Therefore, ELISPOT assays performed on pleural fluid mononuclear cells may be useful for diagnosis of TBP (11). The present study evaluated the performance of an M. tuberculosis antigen–specific IFN-γ ELISPOT assay developed in house on pleural fluid mononuclear cells (PFMC) for the diagnosis of TBP in a setting with a high incidence of TB disease.

MATERIALS AND METHODS

Study population. A prospective study was performed in which 332 consecutive patients with pleural effusion were enrolled at Shenzhen Third People’s Hospital from March 2009 to July 2010. The study was approved by the Institutional Review Board of Shenzhen Third People’s Hospital. Written informed consent was obtained from all participants. All patients had a medical history taken and physical examination performed, with routine investigations including testing for HIV infection, chest radiography, microbiological sputum examination whenever possible, and aspiration of up to 50 ml (15 to 50 ml) of pleural fluid for biochemical, cytological, and detailed microbiological evaluation. Heparinized whole blood (10 ml) and pleural fluid (15 to 50 ml) were simultaneously collected from patients for ELISPOT assay. Patients with HIV infection were excluded.

Diagnosis of tuberculous pleurisy. The diagnosis of TBP was confirmed if a patient had an exudative effusion and was culture positive for M. tuberculosis (using pleural fluid, a pleural biopsy specimen, or sputum) and/or had evidence of TB based on pleural biopsy specimens positive for granulomatous inflammation with acid-fast bacilli (AFB) present (12–14). Non-TB patients were defined on the basis of having no microbiological or histological evidence of TB or no confirmation of an alternative
TABLE 1 Characteristics of study population.

<table>
<thead>
<tr>
<th>Patient characteristic</th>
<th>TBP</th>
<th>Non-TBP</th>
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<tbody>
<tr>
<td>n</td>
<td>281</td>
<td>30</td>
</tr>
<tr>
<td>Age, yr (mean ± SD)</td>
<td>34.1 ± 13.9</td>
<td>52.9 ± 17.7</td>
</tr>
<tr>
<td>Sex (no. F/no. M)</td>
<td>82/199</td>
<td>13/17</td>
</tr>
<tr>
<td>Total cell count</td>
<td>319.5 ± 2,418.8</td>
<td>3,560.8 ± 3,183.9#</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>79.3 ± 13.8</td>
<td>64.0 ± 20.0*</td>
</tr>
<tr>
<td>Total protein (U/liter)</td>
<td>52.8 ± 8.5</td>
<td>45.6 ± 11.7*</td>
</tr>
<tr>
<td>ALP (U/liter)</td>
<td>36.5 ± 13.3</td>
<td>50.2 ± 34.7*</td>
</tr>
<tr>
<td>LDH (U/liter)</td>
<td>445.9 ± 301.6</td>
<td>659.37 ± 613.8#</td>
</tr>
<tr>
<td>ADA (U/liter)</td>
<td>36.1 ± 13.3</td>
<td>13.4 ± 7.8*</td>
</tr>
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*All groups compared with TBP group. #, P is not significant; *, P < 0.01.

**PBMC and PFMCL ELISPOT assay.** *M. tuberculosis* antigen-specific IFN-γ production by PBMC was determined by using an in-house ELISPOT assay as previously described (7, 8). Briefly, PBMC from participants were isolated from whole blood by centrifugation over a Ficoll-Hypaque density gradient (Ficoll-Paque Plus; Amersham Biosciences). Cells were resuspended in Lympho-Spot medium (U-CyTech biosciences, the Netherlands) at a final concentration of 2 × 10^6 cells/ml. A total of 2 × 10^5 PFMC/well were used as negative or positive controls, respectively. Biologically active IFN-γ detection monoclonal antibody (eBioscience, USA) was added for 4 h, followed by the addition of streptavidin-alkaline phosphatase conjugate (Pierce Biotechnology, USA) for 1 h. After a washing step, nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma, USA) chromogenic substrate was added. The individual spots were counted by using an automated image analysis system ELISPOT reader (BioReader 4000 Pro-X; Biosys, Germany).

For PFMCL ELISPOT assay, 15 to 50 ml of pleural fluid was collected to obtain PFMCL by centrifugation at 300 × g for 5 min. The sediment was suspended with 5 ml of red blood cell lysis solution (MilenyiBiotec GmbH, Germany), allowed to stand for 1 min at room temperature, and then washed with 5 ml of RPMI 1640 containing 10% fetal bovine serum (FBS). This was centrifuged at 300 × g for 5 min, and the cells were resuspended with 5 ml of RPMI 1640 containing 10% FBS. Ten microliters of this suspension was mixed with 90 μl of trypan blue solution to evaluate viability and to count cells. The cells were resuspended in Lympho-Spot medium (U-Cy-Tech biosciences, the Netherlands) at a final concentration of 2 × 10^6 cells/ml. A total of 2 × 10^5 PFMC/well were used for ELISPOT assays using the same protocol as for ELISPOT with PBMC as described above.

The numbers of spot-forming cells (SFCs) from PBMC and PFMCL that resulted from no stimulation were subtracted from the responses of PBMC and PFMCL to ESAT-6 protein or ESAT-6/CFP-10 stimulant; a positive response was declared when net SFCs in response to ESAT-6 protein and/or SFCs in response to an ESAT-6/CFP-10 peptide pool were over the cutoff value as determined by receiver operating characteristic (ROC) analysis.

**Determination of ADA activity in pleural fluid.** ADA activity levels were detected with an adenosine deaminase measurement kit (Kefang Biological Technology, Guangzhou, China) by following the manufacturer’s instructions.

**Statistical analysis.** Data were analyzed by using GraphPad Prism 3.0 (GraphPad, La Jolla, CA). The difference of *M. tuberculosis* antigen-specific IFN-γ responses between two groups was analyzed by unpaired t test. The Wilcoxon matched-pair t test was used to analyze the *M. tuberculosis* antigen-specific IFN-γ responses in PBMC and in PFMCL of tuberculosis pleurisy patients. The agreement between the different tests was assessed by estimating Cohen’s κ statistic. Differences were considered significant when the P value was less than 0.05. The Pearson r test was used to analyze the correlation between *M. tuberculosis* antigen-specific IFN-γ responses in PBMC and those in PFMCL. ROC analysis was performed to determine the power of ADA and *M. tuberculosis* antigen-specific immune responses to distinguish patients with TBP from those with non-TBP.

**RESULTS**

Study population characteristics. A total of 332 patients with pleural effusions were qualified and recruited for this study. Among them, 281 were diagnosed with TB based on culture or pathology. The remaining 51 patients were confirmed to have pleural effusions that were not caused by *M. tuberculosis* infection (non-TBP). The clinical characteristics of patients are summarized in Table 1. Overall, non-TBP patients were older than TB patients, although the difference was not statistically significant. Higher percentages of lymphocytes, total protein levels, and ADA activity were found in the pleural fluid of TB patients than in that from patients with lung cancer and those with liver cirrhosis, but not those with pneumonia. There was also no difference in total cell counts or alkaline phosphatase (ALP) or lactic dehydrogenase (LDH) activities. Most TB patients also had pulmonary TB, and 23 were sputum smear positive for acid-fast bacilli (AFB) as detected by Ziehl-Neelsen staining. AFB smear microscopic examination was performed on pleural fluid samples collected from 195 cases of TB, and only 2 (1.02%) were positive. In contrast, the prevalence of *M. tuberculosis* in pleural fluid was detected by culture from 32 of 82 (39.0%) TB cases (Table 1). Histopathologically, 79.7% (224/281) of TB patients had caseating granulomas, while the rest of the TB patients had noncaseating granulomas.

**Enrichment of *M. tuberculosis* antigen-specific IFN-γ-producing cells in pleural fluid from patients with TB.** Consistent
with our previous report (8), we found that TBP patients had significantly higher numbers of \textit{M. tuberculosis} antigen-specific IFN-\gamma-producing cells (Fig. 1A and C) in peripheral blood than did patients with non-TBP effusions (Fig. 1B and D). To further address antigen-specific IFN-\gamma responses at the site of infection, we simultaneously determined the numbers of antigen-specific IFN-\gamma-producing cells in PBMC and matched PFMC. In line with previous reports (10, 15), we found that antigen-specific IFN-\gamma-producing cells were significantly increased in PFMC compared with matched PBMC in TBP patients (Fig. 1A and C). As expected, the numbers of antigen-specific IFN-\gamma-producing cells were not different between PFMC and PBMC from non-TBP patients (Fig. 1C and D). The median values of antigen-specific IFN-\gamma spot-forming cells (SFCs) in PFMC stimulated with ESAT-6 protein and an ESAT-6/CFP-10 peptide pool (335 and 346, respectively) were 4- to 5-fold higher than in PBMC (84 and 68, respectively) from the same TBP patients. Furthermore, the correlation coefficients between numbers of SFCs in PFMC and those in PBMC are low (Fig. 2A and B), suggesting local expansion in addition to infiltration of \textit{M. tuberculosis} antigen-specific IFN-\gamma-producing cells in pleural fluid. On the other hand, the numbers of SFCs to ESAT-6 protein are in good correlation with those to the peptide pool both in PBMC and in PFMC, indicating that both protein and peptide can efficiently stimulate IFN-\gamma production in an ELISPOT assay.

**PFMC ELISPOT assay performs significantly better than PBMC ELISPOT assay for the diagnosis of TBP.** The finding that \textit{M. tuberculosis} antigen-specific IFN-\gamma-producing cells are enriched in pleural fluid from TBP patients, but not in that from non-TBP patients, suggested that PFMC ELISPOT assay might be more sensitive for diagnosis of TBP than PBMC ELISPOT assay. As expected, receiver operating characteristic (ROC) analysis demonstrated that PFMC ELISPOT assays have higher areas under the curve (AUC) than PBMC ELISPOT assays (Fig. 3). Based on the ROC data, the optional cutoff values for ESAT-6 protein and the peptide pool in the PFMC ELISPOT assay were set at 86 and 100 SFCs per $2 \times 10^5$ cells, respectively. In a similar way, we set cutoff values for ESAT-6 protein and the peptide pool in the PBMC ELISPOT assay as 40 and 30 SFCs per $2 \times 10^5$ cells, respectively. A positive response was scored as the numbers of SFCs responding either to ESAT-6 protein or to the over cutoff values. Such a combination increased the sensitivity of PFMC ELISPOT assay from 92.9% to 95.7% without compromising its specificity. Similarly, the sensitivity of PBMC ELISPOT assay was also increased from 70.8% to 78.3%, but the specificity was decreased from 94.1% to 86.3% (Table 2). Using these standards, 220 (78.3%) out of 281 patients with TBP were positive by PBMC ELISPOT assay and 269 (95.7%) out of 281 by PFMC ELISPOT assay. Among controls, ELISPOT assay was positive for seven out of 51 (13.7%) non-TBP patients when performed on PBMC and none (0.0%) of 51 when performed on PFMC. The sensitivity, specificity, positive predictive value, and negative predictive value of the ELISPOT assay for the diagnosis of active TBP when performed on the PFMC were 95.7%, 100%, 100%, and 81.0%, respectively. Thus, the performance of the PFMC ELISPOT assay for diagnosis of TBP is significantly improved compared with that of the PBMC ELISPOT assay (Table 2).

While biopsy of pleural tissue is still considered the most reliable method to confirm the diagnosis of TBP, only 32 out 281 TBP patients had been confirmed by pleural fluid \textit{M. tuberculosis} culture. Therefore, we further evaluated the performance of the PFMC ELISPOT assay in diagnosis of culture-confirmed TBP...
(n = 32) and non-culture-confirmed TBP (n = 249); patients were either positive by pleural fluid culture or diagnosed as positive without a culture test for M. tuberculosis. Thirty-one out of 32 (96.9%) culture-confirmed TBP and 238 out of 249 (95.6%) non-culture-confirmed TBP cases were positive by the PFMC ELISPOT assay. The sensitivity with culture-confirmed TBP was slightly higher than with non-culture-confirmed TBP, but not statistically different, possibly reflecting the higher bacterial antigen load in the former group (8). Taken together, these results confirmed the high sensitivity of PFMC ELISPOT assay in diagnosis of TBP.

Comparison of the PFMC ELISPOT assay and pleural fluid ADA activity test for the diagnosis of TBP. The measurement of ADA activity in pleural fluid is currently a preferred approach for the diagnosis of TBP, offering superior specificity and sensitivity compared to those of other commonly used clinical laboratory tests (3,16). We therefore compared the performance of the PFMC ELISPOT assay with that of the ADA activity test in our

![Graphs of correlation analysis between M. tuberculosis antigen-specific IFN-γ responses to ESAT-6 protein and those to the ESAT-6/CFP-10 peptide pool in PBMC and matched PFMC from TBP patients (n = 281).](http://cvi.asm.org/)

FIG 2 Correlation analysis between M. tuberculosis antigen-specific IFN-γ responses to ESAT-6 protein and those to the ESAT-6/CFP-10 peptide pool in PBMC and matched PFMC from TBP patients (n = 281). (A) Correlation analysis between the numbers of SFCs to ESAT-6 in PBMC (x axis) and in PFMC (y axis), (B) Correlation analysis between the numbers of SFCs to ESAT-6/CFP-10 peptides in PBMC (x axis) and in PFMC (y axis), (C and D) Correlation analysis between the numbers of SFCs to ESAT-6 (x axis) and to ESAT-6/CFP-10 peptides (y axis) in PBMC (C) and in PFMC (D). The Pearson t test was used to analyze the correlation. The correlation coefficients (r) and P values are indicated.

![Graphs of discrimination between TBP and non-TBP cases.](http://cvi.asm.org/)

FIG 3 M. tuberculosis antigen-specific IFN-γ response in PFMC ELISPOT assays is useful for diagnosis of TBP. ROC analysis was performed to determine the power of ADA, ESAT-6 protein-specific SFCs in PFMC, ESAT-6/CFP-10 peptide pool-specific SFCs in PFMC, and ESAT-6 protein-specific SFCs and ESAT-6/CFP-10 peptide pool-specific SFCs in PBMC to distinguish TBP from non-TBP. 95% CI, 95% confidence interval.
A cohort of TBP and non-TBP patients. A total of 156 out of 281 TBP patients and 7 of 51 patients with non-TBP were positive for ADA activity at the cutoff value of 35 U/liter (Table 2). The sensitivity and specificity of ADA were therefore 55.5% and 86.3%, respectively. The agreement between the ADA activity test and PFMC ELISPOT assay was poor (69.4%; κ = 0.27), with significantly higher sensitivity found for PFMC ELISPOT than for pleural fluid ADA activity testing (Table 2).

### DISCUSSION

To our knowledge, there were limited publications on evaluating the performance of antigen-specific IFN-γ for diagnosis of TBP (11, 17–20), with conflicting results, possibly due to the small number of subjects recruited for evaluation (n = 36, n = 66, n = 63, n = 40, and n = 97, respectively). Our study provided the largest cohort so far and confirmed that antigen-specific PFMC ELISPOT assay was useful for diagnosis of TBP with high confidence. We showed that the PFMC ELISPOT assay had a sensitivity of 95.7% and a specificity of 100%. The sensitivity of the PFMC ELISPOT assay was significantly higher than that of the PBMC ELISPOT assay, consistent with compartmentalization of the antigen-specific immune response at the site of disease. The sensitivity of our in-house ELISPOT assay with PFMC is comparable to antigen-specific immune response at the site of disease. The sensitivity of the PFMC ELISPOT assay was significantly higher than that of the PBMC ELISPOT assay (protein + peptide) (86.3% and 96.9%, respectively). Our study provided the largest cohort so far and confirmed that antigen-specific PFMC ELISPOT assay had significantly higher sensitivity and specificity for diagnosis of TBP than the PBMC ELISPOT assay by subtracting the background from SFCs to ESAT-6 protein or ESAT-6/CFP-10 stimulant. This is due to enrichment of M. tuberculosis antigen-specific IFN-γ-producing cells in pleural fluid in patients with TBP but not in those with non-TBP effusions. In line with this, recent observations showed that CD4+ T cells, but not CD8+ T cells, are significantly increased in pleural fluid compared to peripheral blood, and detection of purified protein derivative (PPD)-specific IFN-γ tumor necrosis factor alpha-positive (TNF-α+) CD4 T cells in pleural fluid provides superior diagnostic accuracy compared to that of peripheral blood cells for TBP (15). Antigen-specific IFN-γ ELISPOT assays on mononuclear cells isolated from bronchoalveolar and cerebrospinal fluid also showed advantages over the PBMC ELISPOT assay in diagnosis of pulmonary TB disease in immunocompromised patients with negative AFB smears and for the diagnosis of patients with tuberculous meningitis, respectively (21, 22).

Biochemical and immunological tests of pleural fluid, including measurement of ADA activity and IFN-γ protein levels in pleural fluid, are routinely used for the diagnosis of TBP because they are simple and relatively inexpensive. However, these tests lack specificity for TB and may be elevated with effusions due to other causes. Dheda et al. (18) reported that measurement of nonspecific (i.e., unstimulated) IFN-γ levels in pleural fluid had even higher sensitivity (97%) and specificity (100%) than the T-SPOT.TB assay on PMNC (86% and 60%, respectively). A previous meta-analysis of 13 published articles showed that the sensitivity of antigen-nonspecific IFN-γ test in pleural fluid for diagnosing TBP ranged from 57% to 100% and the specificity from 90% to 100% (23). Thus, the performance of antigen-nonspecific IFN-γ testing may vary substantially dependent on applications in different settings. Nevertheless, considering that the test is simple and economically feasible, it is still important from a public health viewpoint to evaluate its clinical diagnostic value in developing countries with a high incidence of TB, such as China.

While the pleural fluid ADA activity test has been widely used for diagnosis of TBP with high sensitivity and specificity (16, 24), the diagnostic value of this test was limited in our hands, with a sensitivity of only 55.5% and a specificity of 86.3% at a cutoff level for ADA of 35 U/liter. The reason underlying the low sensitivity of ADA testing in our study may be that most of our specimens were collected from TBP patients diagnosed by a positive pleural bi-
opsy. A similar situation has been reported by Muranishi et al. (25). Consistently, our data showed that ADA in pleural fluid collected from patients with positive M. tuberculosis cultures (40.4 ± 10.7 U/liter) was always greater than in pleural fluid from those with negative cultures (31.2 ± 13.6 U/liter). Twenty-four out of 32 (75%) TBP patients with positive M. tuberculosis culture were ADA positive at a cutoff value of 35 U/liter, while only 22 out of 50 (44%) TBP patients with negative culture were positive.

Another reason underlying the low performance of ADA in our study is most likely the high ADA levels in pleural fluid of patients with parapneumonic effusion. A variety of pleural conditions lead to an elevated pleural fluid ADA level (4, 14, 26), with parapneumonic effusion and empyema being probably the most important. In line with this, previous reports (13, 14, 24) showed that roughly 1/10 to 1/3 of parapneumonic effusions and 1/2 to 2/3 of empyemas have ADA levels above 40 U/liter. In this study, the ADA level (38.1 ± 19.2 U/liter) in patients with parapneumonic effusion was not only significantly higher than in the other two control groups (lung cancer, 13.4 ± 7.8 U/liter, and liver cirrhosis, 11.8 ± 14.1 U/liter) but also even higher than that in patients with TBP (36.1 ± 13.5 U/liter) (Table 1). Five out of 8 patients with pneumonia were positive for ADA in the pleural fluid at the cutoff value of 35 U/liter. Accordingly, when these 8 patients with parapneumonic effusion were excluded from the non-TBP control group, ROC analysis showed a sensitivity of 85.1% and a specificity of 90.7% for ADA, which were comparable to the accuracy of ADA from those studies performed in China with the aim of evaluating the performance of ADA in discriminating tuberculous pleural effusion from malignant pleural effusion (27–30).

One limitation of this study is that we can exclude infection with nontuberculous mycobacteria (NTM) in our TBP patients, although this is a rare phenomenon (31, 32), since the presence of AFB in tissue specimens cannot differentiate M. tuberculosis from NTM and most of our TBP patients (249/281) either were negative for pleural fluid M. tuberculosis culture or were not tested by pleural fluid culture. Nevertheless, we routinely performed a 16S rRNA gene-derived probe hybridization assay (Yanbang Biotechnology Co., Shenzhen, China) to identify M. tuberculosis and other NTM among clinical M. tuberculosis isolates. Using this method, we found none of 32 mycobacterial isolates cultured from pleural samples in this study to be NTM. In addition, more than 90% of cases had positive ELISPOT assay responses in PFMC to M. tuberculosis antigens that are not expressed by NTM. We are therefore confident that the cases reported in our study were TBP.

In conclusion, the M. tuberculosis antigen-specific IFN-γ ELISPOT assay (using ESAT-6 protein or a pool of ESAT-6 and CFP-10 peptides) when performed on pleural fluid mononuclear cells holds promise as a useful and rapid diagnostic test for TBP.

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