Several biological markers have been proposed to improve the efficacy of diagnosing tuberculous pleurisy. The study was undertaken to evaluate the accuracy of pleural fluid adenosine deaminase (ADA) activity and interferon-gamma (IFN-γ) concentration in differentiating tuberculous pleural effusion (TPE) and non-tuberculous pleural effusion (non-TPE). Ninety four patients (50 M and 44 F, mean age 60±18, range 18-95 years) with pleural effusion (PE) were studied. TPE was diagnosed in patients with: (i) positive pleural fluid or pleural biopsy culture or (ii) granulomas in the pleural biopsy specimen, after exclusion of other granulomatous diseases. Pleural fluid ADA activity was measured with the colorimetric method of Giusti, while IFN-γ level was measured with ELISA. TPE was diagnosed in 28 patients. The non-TPE group consisted of 35 patients with malignant PE, 20 patients with parapneumonic effusion/pleural empyema, 5 with pleural transudate, and 6 with miscellaneous PE. The ADA activity and IFN-γ concentration were significantly higher in TPE than in non-TPE (614.1±324.5 vs. 15.1±36.0 pg/ml, P<0.0001 and 75.1±39.1 vs. 11.0±16.6 U/l respectively, P<0.0001). The diagnostic sensitivity and specificity of IFN-γ measurement (cut-off value of 75.0 pg/ml) were 100% and 98.5% respectively and were similar to those of ADA (100% and 93.9% at the cut-off value of 40.3 U/L). We conclude that pleural fluid ADA activity and IFN-γ concentration are highly sensitive and specific markers of tuberculous pleurisy.

Key words: adenosine deaminase, interferon-gamma, pleural effusion, tuberculous pleural effusion, tuberculous pleurisy
INTRODUCTION

Despite the introduction and popularity of the new methods of *Mycobacterium tuberculosis* detection and identification, the diagnosis of some cases of tuberculosis, continues to pose considerable difficulty. Tuberculous pleurisy - the most common form of extrapulmonary tuberculosis in Poland - may serve as an example. Since tuberculous pleural effusion (TPE) usually contains a low number of mycobacteria, the diagnostic sensitivity of both direct microscopy and pleural fluid cultures is relatively low (1). A better diagnostic efficacy is provided by examination of pleural tissue samples. The collection of pleural tissue, however, requires more invasive procedures, such as percutaneous pleural biopsy or thoracoscopy. The relatively uncommon use of these diagnostic techniques may be responsible for a surprisingly low number of patients with TPE, compared with all new cases of tuberculosis reported in Poland. The percentage of tuberculous pleurisy among all newly reported cases of tuberculosis in Poland ranged between 3.4% in 2006 and 4.0% in 2004 (data published by National Research Institute of Tuberculosis and Lung Diseases, Warsaw, Poland). In this situation, a variety of biological markers have been proposed to help in the diagnosis of tuberculous pleurisy. They include pleural fluid level of: interferon-gamma (IFN-γ), adenosine deaminase (ADA), soluble interleukin-2 receptor (sIL-2R), immunosuppressive acidic protein (IAP), and others (2, 3). The aim of the present study was to evaluate the usefulness of pleural fluid ADA and IFN-γ measurements in the differentiation between tuberculous and non-tuberculous pleural effusions (non-TPE).

MATERIAL AND METHODS

The study protocol was approved by the Institutional Ethics Committee. The study was conducted in 118 patients with pleural effusion admitted to the Department of Internal Medicine, Pneumonology and Allergology, Warsaw, Medical University in Warsaw, Poland, between 2003 and 2006. The causative evaluation of the pleural effusion was carried out in accordance with the widely accepted algorithm presented in Fig. 1.

All patients underwent thoracentesis and routine pleural fluid analysis, including the measurements of physicochemical parameters (specific gravity, pH, protein, LDH, and glucose) and total and differential cell counts. Effusions were classified as transudates or exudates using Light's criteria (and serum-effusion albumin gradient criterion in some doubtful cases of exudates) (4). This required determination of a total protein concentration and LDH activity not only in pleural fluid, but also in peripheral blood samples. In the majority of patients, cytological and microbiological examinations of pleural fluid were performed. The methods for detecting acid-fast bacilli in pleural fluid included: direct microscopy of Ziehl-Neelsen stained slides, Lowenstein-Jensen culture, and, in some cases, detection of *M. tuberculosis* DNA sequences by an amplification test (Amplicor *Mycobacterium tuberculosis* (MTB) (Roche Diagnostics, USA). Pleural fluid samples (mean volume 50 ml) for ADA and IFN-γ measurements were centrifuged at 2000 revolutions per minute for 10 min and the supernatant was frozen at -70°C. Additional diagnostic procedures, such as chest computed tomography, bronchoscopy, echocardiography, and others were undertaken, whenever
necessary, to further evaluate pleural fluid etiology. Pleural biopsies were subjected to histologic and microbiological evaluation (identification of acid-fast bacilli, methodology identical to that described for pleural fluid).

Based on the above tests we identified a group of 94 patients with a determined pleural fluid etiology. This group included patients with tuberculous pleurisy, malignant pleural effusion, parapneumonic effusion and/or pleural empyema, pleural transudates caused by heart failure, and a group of patients with miscellaneous, less frequent causes of pleural effusion.

**Definitions**

At least one of the following criteria had to be met to diagnose tuberculous pleural effusion: (i) positive culture for *M. tuberculosis* in pleural fluid, pleural biopsy, and fibrinous adhesions collected during thoracoscopy or respiratory samples (sputum, bronchial washing, BALF) and (ii) caseating granulomas in pleural biopsy samples (3).

Malignant pleural effusion was diagnosed in patients with: (i) positive pleural fluid cytology and/or positive histology of pleural biopsy and (ii) a known malignant disease, after the exclusion of alternative causes of pleural effusion (5).

Parapneumonic effusion or pleural empyema was diagnosed in patients who had: (i) grossly purulent pleural effusion, (ii) the presence of microorganisms in pleural fluid, or (iii) sign and symptoms of pneumonia accompanied by pleural effusion which resolved following antibiotic treatment and/or local pleural drainage (6).

Pleural transudate was diagnosed according to Light's criteria. The specific etiology of transudative effusions (congestive heart failure, liver cirrhosis, and nephrotic syndrome) was based on clinical and laboratory data and a negative cytology and microbiology of the pleural fluid (7). All pleural effusions caused by less common underlying condition was classified as a group of miscellaneous pleural effusions (e.g., post-by pass surgery syndrome, trapped lung, and pulmonary embolism).

The pleural fluid ADA activity was determined by colorimetry according to the method of Giusti (8), in accordance with the previously described protocol (9). The measurement of IFN-γ concentration in pleural fluid samples was performed by an immunoenzymatic method using a

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**Fig. 1. Diagnostic algorithm in patients with pleural effusion.**
commercial kit (Quantikine Human IFN-γ Immunoassay, R&D Systems, USA). The range of measurable concentrations of IFN-γ in undiluted samples ranged from 8 to 1000 pg/ml. If the IFN-γ concentration exceeded the lower or the upper limit of measurement, the lowest or the highest measurable value was used.

Statistical analysis was performed using a statistical software package (StatSoft, Inc. STATISTICA, version 8.0, www.statsoft.com.). Data are presented as means ±SD and ranges. The non-parametric, Mann-Whitney U-test was used to test for significance between different groups. A P<0.05 was regarded significant. Receiver operating characteristics (ROC) curves were constructed and analyzed to determine the most accurate cut-off values for the diagnosis of tuberculosis.

RESULTS

Tuberculous pleurisy was diagnosed in 28 patients, while non-tuberculous pleural effusion (non-TPE) was diagnosed in 66 patients. Comparative characteristics of patients with TPE and non-TPE are presented in Table 1.

More detailed characteristics of patients with non-TPE are presented in Table 2. This group included 35 patients with malignant pleural effusion, 20 patients with parapneumonic effusion/pleural empyema, 5 patients with transudative effusion caused by heart failure, and 6 patients with miscellaneous underlying conditions (2 patients with post by-pass surgery syndrome, 1 patients with post-traumatic pleural effusion, 1 patient with spontaneous pneumothorax associated pleural effusion, and 1 with asbestos pleurisy).

The microbiological studies of 28 pleural fluid samples collected from patients with tuberculous pleurisy detected *Mycobacterium tuberculosis* or its nucleic acid sequences in 10 samples (10/28, 35.7%). Sensitivity of the individual methods for identification of microorganisms in pleural fluid were as follows: 2/28 positive results using direct microscopy (sensitivity: 7%), 10/28 positive results using culture (sensitivity 36%) and 4/28 positive results using the PCR method (sensitivity 14%). In 24 (85.7%) patients typical granulomas in pleural tissue

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tuberculous pleural effusion (n=28)</th>
<th>Non-tuberculous pleural effusion (n=66)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>46.7 ± 20.1</td>
<td>64.0 ± 14.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex M/F</td>
<td>19/9</td>
<td>31/35</td>
<td></td>
</tr>
<tr>
<td>Body temperature (°C)</td>
<td>38.1 ± 0.9</td>
<td>37.6 ± 0.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PPD (mm)</td>
<td>12.5 ± 7.5</td>
<td>4.7 ± 6.7</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Pleural fluid protein concentration (g/dL)</td>
<td>5.2 ± 0.6</td>
<td>4.0 ± 1.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pleural fluid LDH (IU/l)</td>
<td>2641 ± 3920</td>
<td>3202 ± 8635</td>
<td>NS</td>
</tr>
<tr>
<td>Pleural fluid total cell count (cells/mm³)</td>
<td>2738 ± 1882</td>
<td>3999 ± 13674</td>
<td>NS</td>
</tr>
<tr>
<td>Pleural fluid lymphocyte percentage (%)</td>
<td>86 ± 18</td>
<td>55 ± 29</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pleural fluid lymphocyte count (cells/mm³)</td>
<td>2374 ± 1622</td>
<td>1232 ± 1648</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pleural fluid ADA (U/l)</td>
<td>75.3 ± 39.1</td>
<td>11.0 ± 16.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pleural fluid IFN-γ concentration (pg/ml)</td>
<td>614.1 ± 324.5</td>
<td>15.1 ± 36.0</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of patients with tuberculous vs. non-tuberculous pleural effusion.
samples were found (18 biopsies were obtained during percutaneous procedure and 6 during thoracoscopy). Microbiological examination of the sputum was performed in 12 patients (positive result in 4 patients). Sixteen patients underwent bronchoscopy. *M. tuberculosis* was found in 5 out of 13 patients who underwent bronchial wash (BW). The examination of the bronchoalveolar lavage fluid (BALF) collected from 3 patients did not reveal the presence of *M. tuberculosis*.

Both ADA activity and IFN-γ concentration were significantly higher in tuberculous versus non-tuberculous effusions (75.3 ±39.1 vs. 11.0 ±16.6, P<
0.0001, and $614.1 \pm 324.5 \text{ pg/ml vs. } 15.1 \pm 36.0 \text{ pg/ml, } P<0.00001$, respectively) (Fig. 2 and Fig. 3).

The highest ADA activity and IFN-γ concentration among the non-tuberculous effusions were observed in parapneumonic effusions/pleural empyemas ($19.6 \pm 25.3 \text{ U/l and } 23.6 \pm 62.9 \text{ pg/ml}$, respectively). These values were, however, significantly lower than those in tuberculous exudates ($P<0.0001$). A relatively high activity of ADA in pleural effusion was also observed in 6 patients with miscellaneous effusions, however, the concentration of IFN-γ in those 6 patients was very low ($8.0 \pm 0.0 \text{ pg/ml}$). An analysis of the ROC curve for ADA activity showed that at the
most accurate cut-off level of 40.3 U/L the sensitivity of the test for tuberculous pleurisy was 100% and the specificity 93.9% (Fig. 4A). Assuming 75 pg/ml as the threshold value for IFN-γ concentration, the diagnostic sensitivity for tuberculous pleurisy was 100% with the corresponding specificity of 98.5% (Fig. 4B).

**DISCUSSION**

Tuberculous pleurisy is one of the most common forms of extrapulmonary tuberculosis. In Poland, between 2003 and 2006, the reported cases ranged from three to four hundred per year. Tuberculous pleural effusion was responsible for more than 40% of all forms of extrapulmonary tuberculosis and ranked first among the extrapulmonary locations of the disease (10). According to the data collected in the USA in late 1980s, tuberculous pleurisy was the second most frequent location of extrapulmonary tuberculosis.

Epidemiological data on the prevalence of tuberculous pleurisy in all patients with pleural effusion are more diverse. In one Spanish study, tuberculosis was the most common cause of pleural effusion (11). On the other hand, studies by Marell et al (12) demonstrated that in a well-defined region of Central Bohemia not a single case of tuberculous etiology was noted in 142 patients with pleural effusion (12). In patients treated in the Department of Internal Medicine, Pneumonology and Allergology, Warsaw Medical University in Warsaw, Poland, tuberculous pleurisy accounted for 11.5-15.0% of all cases of pleural effusion (data from 2004 to 2006). This variability in the prevalence of pleural tuberculosis is undoubtedly a result of the local epidemiological situation, the profile of the center where the study was conducted, and considerable variations in the sensitivity of the diagnostic procedures employed to confirm the tuberculous etiology of effusion. It is well known that because of the low number (or even absence) of *M. tuberculosis* in pleural fluid, sensitivity of various methods of *M. tuberculosis* identification in pleural fluid is low (0-5% for direct microscopy and 25-37% for culture-based methods) (1, 13). The highest diagnostic sensitivity, reaching 70-80%, has been observed for PCR methods identifying specific nucleic acid sequences in pleural fluid. These diagnostic methods are, however, associated with a relatively high rate of false positive results (specificity of about 90%) (4, 13). The sensitivities of the individual methods for *M. tuberculosis* identification which were found in our study are consistent with the above cited data, with the exception of the PCR method. In our study, the sensitivity was 7% (2/28) for direct microscopy, 35.7% (10/28) for culture, and only 14.3% (4/28) for the PCR method. It is noteworthy that in all four patients with positive PCR results, the presence of *M. tuberculosis* was also confirmed by other methods (positive culture in all four patients, positive direct microscopy in two of these patients). Therefore, PCR played an essential diagnostic role only in 2 patients, in whom it gave a rapid confirmation of the tuberculous etiology of pleural effusion. A comparison of some demographic and
clinical data found in patients with tuberculous and non-tuberculous effusions was consistent with other author's observations in that the mean age of patients with TPE was significantly lower than that of patients with non-TPE (P<0.0001) and the mean body temperature was significantly higher in subjects with TPE than that in the remaining group of patients (P<0.05).

Our results draw attention to the well recognized fact that biopsy methods play a crucial role in the diagnostic algorithm in patients with TPE (14). The sensitivity and specificity of these methods are so high that they are often recommended as a diagnostic 'gold standard' in patients with tuberculous pleurisy. Had these methods not been employed in our study group, the diagnostic sensitivity with respect to tuberculous pleurisy would have been 46.4% (13/28). Therefore, we believe that the histological and microbiological evaluations of pleural tissue samples are necessary to reliably estimate the potential usefulness of a new diagnostic methods in patients with tuberculous pleurisy. In our study percutaneous pleural biopsy was the most common procedure used for this purpose (15).

The search for effective but less invasive methods to accurately diagnose TPE has been going on for years. Several biological markers measured in pleural fluid have been found to be sensitive and relatively specific indices of tuberculous pleurisy. These markers include: adenosine deaminase, IFN-γ, and other cytokines or substances detectable in TPE (e.g., IL-6, IL-12, IL-18, soluble IL-2 receptor, TNF-α) (2, 3, 9, 16, 17).

Adenosine deaminase is an enzyme involved in the conversion of adenosine to inosine. Two isoenzymes of ADA have been identified. They are known as ADA1 and ADA2. ADA1 is present in nearly all body cells. ADA2 is mainly expressed in monocytes and macrophages and is released to the extracellular space following stimulation of these cells triggered by intracellular infection. Valdes et al (18) demonstrated that the activity of both isoenzymes contributes to the high ADA activity in tuberculous pleural fluids, with ADA2 playing the predominant role. Elevated ADA activity is not specific for TPE, but is also seen in parapneumonic effusions and pleural empyemas, rheumatoid effusions, and certain malignant pleural effusions.

A meta-analysis of 31 studies published between 1978 and 2000, which assessed the value of pleural fluid ADA activity in differentiation between TPE and non-TPE demonstrated a high sensitivity and specificity of these measurements (92 and 89%, respectively) (19). In over 2/3 of these studies ADA activity was measured with a colorimetric method described by Gusti (8). Although the cut-off value applied by different authors ranged between 10 and 70 U/L, the mean ADA cut-off value in 31 analyzed studies was very close to the best discriminating value in our study (41.8 and 40.3 U/L, respectively). With a cut-off level of 40.3 U/L estimated from ADA ROC analysis, we found very high diagnostic sensitivity and specificity of the test (100 and 93.9%), respectively. Similarly to the previous studies, the sensitivity of ADA determinations in our study proved to be slightly lower that its specificity. Four patients with non-
tuberculous pleural effusion had ADA activity exceeding threshold level 40.3 U/L. This group included 3 patients with parapneumonic exudate/pleural empyema and 1 patient with benign asbestos pleurisy. In order to increase the specificity of the assay some authors recommend that the diagnostic measurements of ADA activity should be limited to lymphocytic exudates. This assumption largely reduces the number of false positive results in patients with parapneumonic exudates.

One cannot ignore the fact that the results of some other authors slightly differ from the results cited above. Zaric et al (20) found that the diagnostic sensitivity of ADA determination in tuberculous pleural fluid was 89.2%, but the specificity was only 70.4%. One reason for this discrepancy may be the differences in the methodology of ADA activity measurements. The results of ADA activity in tuberculous pleural exudate are probably also affected by racial differences; the test is less useful in the yellow race.

In the most recent meta-analysis of studies investigating the use of pleural fluid ADA activity for the diagnostic evaluation of TPE, in which a total of 63 studies have been analyzed, sensitivity was estimated at 92% and specificity at 90% (21). Daniil et al (22) recently showed that the diagnostic accuracy may be improved when ADA activity in pleural fluid is analyzed together with the C-reactive protein (CRP) concentration in pleural fluid.

The practical role of ADA measurement in discriminating between TPE from non-TPE depends not only on clinical context, but also on local epidemiological situation. In low and intermediate tuberculosis prevalence, a negative predictive value (NPV) plays the most important role. In these settings, low ADA activity in pleural effusion, with a very high probability, excludes tuberculous pleural fluid etiology. In contrast, in populations with high tuberculosis prevalence the high sensitivity of the test should be used. In those settings, high ADA activity provides very high post-test probability of pleural tuberculosis (19).

Another highly sensitive and specific marker of the tuberculous pleurisy is IFN-γ concentration in pleural fluid (2, 13, 23). The increased IFN-γ concentration in tuberculous exudates can be explained by the cellular mechanisms of immune reactions to infection with \textit{M. tuberculosis}. Expression of \textit{M. tuberculosis} antigens on the surface of antigen-presenting cells leads to T cell activation, increased T cell count, and release of cytokines that play a role in the further stages of the immune response. These cytokines include IL-2 and IFN-γ. Since in the chronic phase of tuberculous pleurisy T cells are the predominant cells in pleural effusion and they are an abundant source of IFN-γ, a high concentration of IFN-γ in TPE can easily be explained (24, 25).

Redistribution of T and B cells and the stimulation of T cells present in the pleural cavity explain the differences between the concentration of IFN-γ in pleural fluid and peripheral blood. One of the studies demonstrated that median IFN-γ concentration in pleural effusion was over 60 times higher than that in the blood (26).

Evidence of the markedly increased IFN-γ concentration in tuberculous pleural effusions has generated obvious interest in this substance as a potential...
diagnostic tool. Results of studies performed in the 1980s showed that pleural fluid IFN-\(\gamma\) measurements for the diagnosis of tuberculous pleurisy are characterized by excellent sensitivity and specificity (100%) (27). Subsequent studies conducted in the 1990s confirmed the high sensitivity, specificity, and diagnostic accuracy of IFN-\(\gamma\) concentration measurements in the diagnosis of TPE, although the results were not as excellent as the results of the previously cited studies (28-31). The sensitivity and specificity of IFN-\(\gamma\) measurements as a diagnostic marker of tuberculous pleurisy ranged from 85.7 to 100%, and from 95 to 97%, respectively. The diagnostic accuracy was estimated between 92.4 and 97.5%. The methodology of IFN-\(\gamma\) determination used in most of the studies was similar and based on using commercial immunoenzymatic assays (ELISA). Only a few centres used radioimmunoassays (RIA) to measure IFN-\(\gamma\) concentration in pleural effusions. Kits from different manufacturers differ in terms of the testing ranges. We used a kit with a testing range from 8 to 1000 pg/ml. In nearly 1/3 (9/28) of the patients with tuberculous effusions IFN-\(\gamma\) concentration exceeded 1000 pg/ml. By setting the cut-off value for IFN-\(\gamma\) concentration at 75.0 pg/ml we obtained diagnostic sensitivity of 100%, specificity of 98.5%, negative predictive value of 100%, and positive predictive value of 96.6%. Our results are very similar to those of other authors, although the cut-off values adopted by them were different and ranged from 1.5 to as much as 300 pg/ml (32, 33).

In 2003, a meta-analysis which summarized the results of 13 studies (performed between 1978 and 2000) evaluating the usefulness of IFN-\(\gamma\) determination for the diagnosis of TPE was published (19). The overall rating of the test effectiveness (sensitivity and specificity expressed by the Q value on the ROC curve) was 96%. Another meta-analysis was published in 2007 (32). It included 11 studies, which were the subject of a previous meta-analysis, and another 11 studies published between 2001 and 2006. A total number of 782 patients with TPE and 1319 patients with non-TPE participated in these 22 studies. The authors placed special emphasis on the quality of the studies and detailed statistical analysis of the results. The meta-analysis confirmed the exceptionally high specificity of the test for the detection of tuberculous pleurisy, which reached 97% (range in the analyzed studies 86 to 100%). Slightly more discrepancies were revealed by the analysis of sensitivity (range 64 to 100%). The overall mean sensitivity was 89%. Of note is that in as many as 10 out of 22 analyzed studies, the specificity of IFN-\(\gamma\) measurement in pleural fluid for the detection of tuberculous pleurisy was 100% and in 5 of them an equally high sensitivity was shown (100%).

The measurement of the intensity of IFN-\(\gamma\) secretion by peripheral blood lymphocytes, following stimulation of \(M.\) \textit{tuberculosis} antigens, is the basis for diagnostic assays characterized by a higher sensitivity and specificity for the detection of latent tuberculous infection, compared with the tuberculin skin test. Studies investigating the usefulness of the measurements of IFN-\(\gamma\) secretion by specifically activated lymphocytes in pleural fluid are currently on-going (34).
In conclusion, ADA activity and IFN-γ concentration are sensitive and specific markers of tuberculous pleurisy. These tests seem to be as useful as the biopsy methods considered the gold standard in diagnosing tuberculous pleural effusion. Measurements of ADA activity and IFN-γ concentration in patients with pleural effusion could reduce the number of patients referred to more invasive diagnostic procedures. They may also decrease the number of patients in whom antituberculous treatment is initiated without confirmation of the tuberculous etiology of the pleural effusion.

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