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Cytokines in pleural liquid for diagnosis of tuberculous pleurisy

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An elevated level of adenosine deaminase (ADA) in pleural liquid has been considered as a supplemental diagnostic marker for tuberculous pleurisy. However, this is complicated by false-positives and -negatives. Recently, it has been revealed that various cytokines are intimately involved in the pathognomonic physiology of tuberculosis. In this study, interleukin-8 (IL-8), tumour necrosis factor α (TNFα) and interferon γ (IFNγ) were compared with ADA in pleural liquid of patients with inflammatory (21 cases), malignant (28 cases) and tuberculous (21 cases) disease. The pleural ADA, IL-8, TNFα and IFNγ levels in the tuberculous group were higher than in the other three groups. Analysis of receiver operating characteristic (ROC) curves, to evaluate the utility of the various parameters, demonstrates values for the area under the curve (AUC) of 0·770, 0·875, 0·892 and 0·987, respectively for IL-8, TNFα, ADA and IFNγ,. No false-positives were encountered with IFNγ and only one case with a small volume of pleural liquid was a false-negative. This indicates that IFNγ is a very reliable marker of tuberculous pleurisy.

Key words: tuberculous pleurisy; IFNγ; TNFα; IL-8; ROC curve.

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Introduction

Making a diagnosis in cases with pleural liquid is not easy sometimes. In most instances, a definite diagnosis can only be established employing histological approaches such as pleural biopsy and thoracoscopy (1,2). For daily clinical activity, however, investigation of markers in pleural liquid is far easier to perform than histological methods. Determination of adenosine deaminase (ADA) in pleural liquid has been in fact considered as a useful supplemental diagnostic index for tuberculous pleurisy. However, the elevation may be limited in early stage disease, and in addition, high levels of ADA can also be found in patients with pyothorax (3,4). Recently, it has been revealed that various cytokines are intimately involved in the pathognomonic physiology of tuberculosis. In this study, interleukin-8 (IL-8), tumour necrosis factor α (TNFα) and interferon γ (IFNγ) were therefore compared with ADA in pleural liquid associated with inflammation, malignancy or tuberculosis. The utility of each marker was evaluated by analysing its sensitivity, specificity and receiver operating characteristic (ROC) curve.

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Patients and methods

PATIENTS

Seventy patients for whom a diagnosis of pleural liquid was well established in either our department or related hospitals between 1993 and 1998 were chosen as subjects. The cytokines IL-8, TNFα and INFγ in pleural liquid, as well as ADA were compared dividing the patients into the three groups detailed below. Pleural liquid was collected before they were given treatments. After centrifugation at 3000 rpm for 5 min, the supernatant was frozen at −80°C. ADA was determined immediately, and cytokines at a late date. The subject groups were:

(1) Inflammatory pleural liquid (21 cases). Pleural liquid which could be ameliorated with the use of antibiotics in patients with no signs of cardiac insufficiency was defined as parapneumonic (14 cases). When the liquid was full of neutrophils and visually purulent this was defined as pyothorax (seven cases). The group consisted of 16 males and five females, with an average age of 74 years.

(2) Malignancy associated pleural liquid (28 cases). Pleural liquid in which the presence of either malignant cells or tissues was verified was defined as malignancy associated (lung cancer, 15; pancreas cancer, two; breast cancer, one;

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renal cancer, one; pleural mesothelioma, one; others, eight). The group consisted of 19 males and nine females, with an average age of 74 years.

(3) Tuberculous pleural liquid (21 cases). Pleural liquid from which tubercle bacilli could be isolated (four cases), or from individuals histologically diagnosed as having tuberculosis (17 cases) was defined as tuberculous. The group consisted of 17 males and four females, with an average age of 68 years.

DETERMINATION OF ADA

The amino radical of adenosine hydrolysed by ADA produces inosine and ammonia. When α-ketoglutaric acid and NADPH are added to the ammonia, L-glutamine and $NADP^+$ are produced due to the reaction of glutaminic-acid dehydrogenase, which reduces the NADPH. This was determined by measuring the reduction in light absorption at 340 nm to evaluate ADA.

DETERMINATION OF CYTOKINES

IL-8 was determined using the ELISA method, based on a two-step sandwich process. Human IL-8 and enzyme-labelled human IL-8 polyclonal antibodies in specimens were sequentially bound to antihuman IL-8 polyclonal antibodies coated at the bottoms of test wells. The concentration of human IL-8 in the specimen was then determined by measuring the activity of the enzyme.

TNFα was determined using an ELISA method, sandwiching with a single layer of antihuman TNFα monoclonal antibodies and POD-labelled antihuman TNFα monoclonal antibodies.

The IFNγ level was determined using a single layer radio-immunoassay again based on a two-step sandwich process. Polystyrene beads coated with human IFNγ-specific mouse monoclonal antibodies were incubated together with specimen and standard solution. After removing combined labelled antibodies, the radioactivity of the beads was counted with a gamma counter. Based on the theory that the radioactivity bound to the beads is proportional to the concentration of IFNγ, the IFNγ concentrations of specimens were determined referring to a standard curve, generated with standard solution.

STATISTICAL METHODS

The data were analysed using the Mann–Whitney method for comparison of means ± standards error. ROC curve analysis was used to determine a cut-off value for making the diagnosis of tuberculosis, and the diagnostic accuracy was evaluated by comparison of each area under the curve (AUC).

Results

The pleural liquid ADA level in the tuberculous group was $72{\cdot}4 \pm 7{\cdot}5\,IU\,l^{-1}$. This was markedly higher than in the other groups, with values of $35{\cdot}0 \pm 8{\cdot}6\,IU\,l^{-1}$ for the inflammatory, and $11{\cdot}2 \pm 1{\cdot}4\,IU\,l^{-1}$ for the malignant group (Fig. 1). The pleural liquid IL-8 level in the tuberculous group was $2398 \pm 862\,pg\,ml^{-1}$, significantly higher than the $540 \pm 298\,pg\,ml^{-1}$ in the inflammatory group, and $193 \pm 52\,pg\,ml^{-1}$ in the malignant group (Fig. 2). TFNα levels were $37{\cdot}8 \pm 11{\cdot}7\,pg\,ml^{-1}$, $9{\cdot}2 \pm 2{\cdot}3\,pg\,ml^{-1}$ and $6{\cdot}3 \pm 0{\cdot}7\,pg\,ml^{-1}$, respectively (Fig. 3). The difference in values for IFNγ was even more pronounced. The level in the tuberculous group was $52{\cdot}5 \pm 11{\cdot}8\,U\,ml^{-1}$, and only $0{\cdot}5 \pm 0{\cdot}2\,U\,ml^{-1}$ in the inflammatory, and $0{\cdot}3 \pm 0{\cdot}1\,U\,ml^{-1}$ in the malignant group (Fig. 4).

ROC curve analysis was conducted in order to compare the usefulness of each cytokine and ADA in making a diagnosis of tuberculosis. The false-positive rate (1-specificity) for each marker was plotted on the horizontal axis,

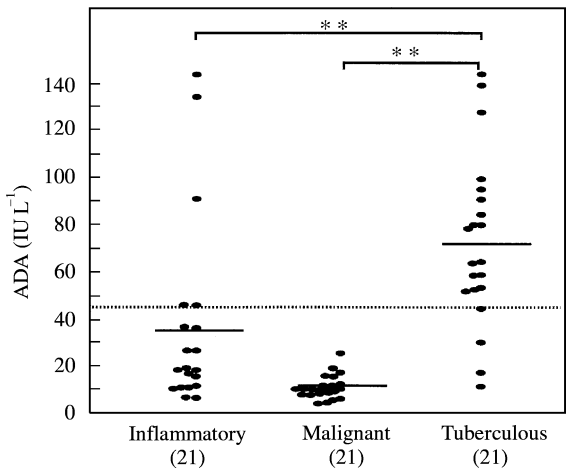

FIG. 1. ADA in pleural effusions. Mean (−), cut-off (- - - -), $*P > 0.01$ (number of subjects in parentheses).

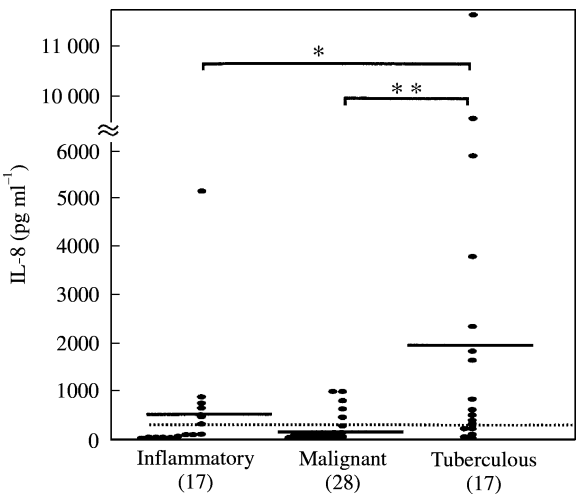

FIG. 2. IL-8 in pleural effusions. Mean (−), cut-off (- - - -), $*P > 0.05$, $**P > 0.01$ (number of subjects in parentheses).

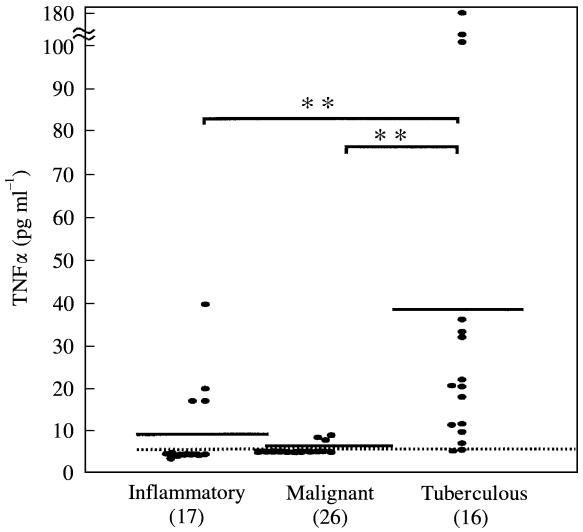

FIG. 3. TNFα in pleural effusions. Mean (−), cut-off (- - - -), *$P>0.\cdot01$ (number of subjects in parentheses).

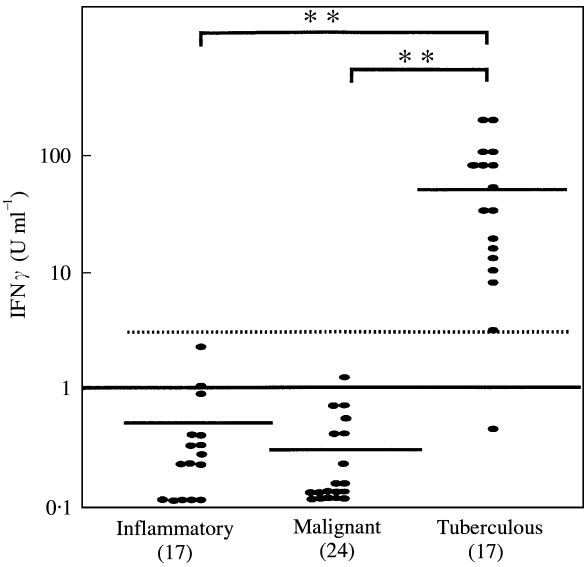

FIG. 4. INFγ in pleural effusions. Mean (−), cut-off (- - - -), *$P>0.01$ (number of subjects in parentheses).

and the positive rate (sensitivity) on the vertical axis (Fig. 5). The AUC value for each parameter was calculated, and the diagnostic accuracy was compared. IL-8 and TNFα showed intermediate accuracies of 0·770 and 0·875, respectively. The results for ADA and IFNγ were both high at 0·892 and 0·987, respectively.

The cut-off for the cytokine was defined when the distance from the point [(1-specificity) = 0, sensitivity = 1] on the ROC curve was at a minimum. For ADA, IL-8, TNFα and IFNγ they were 45·0 IU l^{-1}, 228 pg ml^{-1}, 6 pg ml^{-1} and 3·1 U ml^{-1}, respectively (Table 1). The sensitivity and specificity with IFNγ were 94·1% and 100%, respectively, which were superior to those for

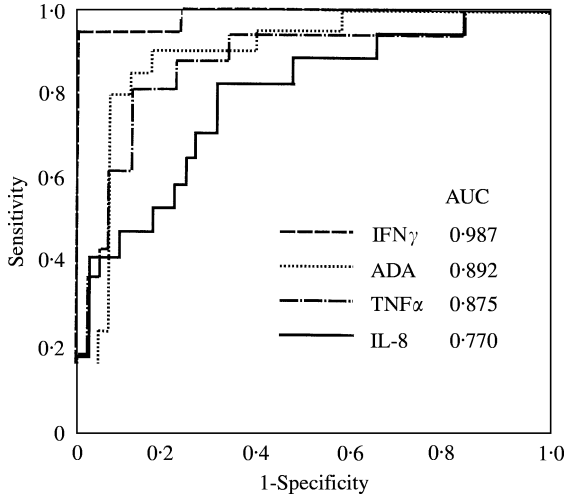

FIG. 5. ROC curves for diagnosis of tuberculosis. AUC: area under the curve.

TABLE 1. Sensitivity and specificity on diagnosis of tuberculous pleural liquid

	IL-8 (pg ml^{-1})	TNFα (pg ml^{-1})	ADA (IU l^{-1})	IFNγ (U ml^{-1})
Ccut-off value	228	6	45	3·1
Sensitivity (%)	82·3	87·5	85·0	94·1
Specificity (%)	72·3	84·0	91·1	100

ADA (sensitivity: 85·0% and specificity: 91·1%), IL-8 (sensitivity: 82·3% and specificity: 72·3%) and TNFα (sensitivity: 87·5% and specificity: 84·0%).

Discussion

The diseases most frequently associated with pleural liquid are tuberculosis, malignant tumours, cardiac insufficiency and bacterial infection. Although various methods such as examination of biochemical conditions, cytodiagnosis, bacteriological searches and biopsy examination are used, cases with an indefinite diagnosis are not rare.

In Japan, tuberculosis has recently been found in approximately 42 000 people per year (1998). In 1998, it became a social issue because an elevation in the prevalence was noted after a long period of decrease. Regarding the accuracy of diagnostic methods for tuberculous pleurisy, the positive rate with smear testing for tubercle bacilli in pleural liquid was less than 10%, that with culturing ranged from 20% to 30%, and the diagnostic rate with cutaneous pleural biopsy ranged from 50% to 80% (5,6). Recently, making a diagnosis at an early stage has been greatly facilitated by detection of tubercle bacilli in pleural liquid with the PCR method. However, the reported range for

positive rates is as wide as 12–100% (7). Pleural liquid ADA has long been used as a marker for tuberculous pleurisy, with sensitivities ranging from 93% to 100%, and specificities from 76% to 100% (4). However, false-positives include cases of pyothorax and other diseases such as lung cancer, lymphoma and pleural mesothelioma, False-negatives may be either in an early stage of tuberculous pleurisy or in a state of insufficient immuinity (6). The results of the present study also showed the ADA level in the tuberculous group to be higher than in the malignancy and inflammatory groups. Although the precise data are not documented in this report, pleural liquid ADA levels in pyothorax cases in the inflammatory group were not different from those in the tuberculous group, and five of six cases presenting as false-positive were suffering from pyothorax. As described previously, ADA seems to be a useful marker of tuberculous pleurisy, excluding pyothorax.

It has been reported that, in patients with tuberculous pleurisy, cell-mediated immunity participates in protection against infection with tubercle bacilli, and IL-1, -8, -10 -12, TNFα and IFNγ are produced (8,9).

In the present study, IL-8, which stimulates T cells, is known to play an important role in granulomatous processes (10) and was particularly elevated in the tuberculous group. Production of IL-8 by monocytes after phagocytizing tubercle bacilli has been described (11), but some researchers reported high levels in pleural liquid associated with either pyothorax or pneumonia, correlating with neutrophil counts and extent of myeloperoxidase activity (12,13). In one report IL-8 elevation was found to be greater with pyothorax or pneumonia than with tuberculosis or malignancy (14). The reasons for the discrepancies are still unclear and may depend on the timing of specimen collection or other factors but clearly, IL-8 is not optimal as a diagnostic marker.

Higher TNFα levels in tuberculous as compared to other groups were found here (15,16). TNFα is considered necessary for production granulomas and removing rod-shaped bacteria in inflammatory lesions, and it is also regarded as an inducer of IFNγ. In rheumatic pleural liquid, TNFα increases in a similar manner as in the tuberculous case. While down-regulation of IFNγ production also occurs in rheumatic pleural liquid (17), a number of researchers have reported that distinction of tuberculous from rheumatic or inflammatory pleural liquid cannot be made with TNFα (17–19).

The IFNγ level itself in the present tuberculous group was markedly higher than in the other groups. When lymphocytes in pleural liquid with tuberculous pleurisy are stimulated by PPD, cytokines, mainly IFNγ, are produced (20), and when they are treated with CD4 monoclonal antibodies and complement, the IFNγ level decreases (17,21). The reason for the increase is considered to be production by $CD4^+$ lymphocytes reacting against tubercle bacilli. In fact, the concentration of tubercle bacilli in pleural liquid correlates with the amount of IFNγ (17). A number of reports have demonstrated that IFNγ levels in patients with tuberculous pleurisy are high, with sensitivities and specificities ranging from 90% to 100% (17,18,20,22–27). Valdes *et al*. conducted research on pleural liquid samples obtained from 145 patients and reported two with small volumes out of 35 tuberculosis cases to be false-negative, while nine out of 110 non-tuberculous cases were false-positives (parapneumonic pleural effusion, three; pulmonary embolism, three; lymphoma, one; lymphocytic leukemia, one; neuroblastoma, one) (11). In our study there were no false-positives with the use of IFNγ, and only one case with a small volume of pleural liquid presented as a false-negative. These findings provide strong support for the conclusion that IFNγ is a reliable marker of tuberculous pleurisy.

ROC curves can profile sensitivity and specificity of markers, and are regarded as useful for analysing and/or comparing diagnostic accuracy (28). While ADA was here found to have good values for both parameters, IFNγ was superior as a marker for tuberculous pleurisy. All of the five cases with false-positive ADA findings were cases with pyothorax, and their IFNγ levels were all lower than the cut-off value. There were three cases showing false-negatives for ADA, but two of these three cases had high IFNγ levels. On these three cases, we got pleural effusion over a month after onset. ADA levels in tuberculous pleurisy may decrease long after onset, but, even if this is also the case for IFNγ, elevation above the cut-off value seems to be maintained.

In conclusion, levels of the pleural liquid cytokines examined in this study were higher in the tuberculous than in the other three groups, IFNγ being shown to be especially useful as a marker of tuberculous pleurisy, with clear advantages over ADA.

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