Evaluation of Cytokines in Pleural Fluid for the Differential Diagnosis of Tuberculous Pleurisy

Amina Kamal EL-Ansary[1] , Mona Ahmed Radwan[2]
[1]Medical Biochemistry* and Internal Medicine
[2]Departments, Faculty of Medicine, Cairo University

ABSTRACT

The present study aims to test reliability of interferon gamma (IFN- γ) as a diagnostic marker for tuberculous pleurisy. In this study, interleukin-8 (IL-8), tumour necrosis factor α (TNF-α) and interferon gamma (IFN-γ) were therefore compared with adenosine deaminase (ADA) in exudative pleural effusion of inflammatory, malignant and tuberculous origin to determine their diagnostic value and whether either or all of them could be helpful in the differential diagnosis of tuberculous pleural effusion. Subjects and methods: This study included 11 patients with inflammatory, 13 patients with malignant, and 15 patients with tuberculous effusion matched for age and sex. Estimation of Pleural fluid levels of ADA, IL-8, TNF-α and IFN-γ. Results: Pleural fluid levels of ADA, IL-8, TNF-α and IFN-γ in the tuberculous group were significantly higher than in the malignant group. Also the levels of pleural fluid ADA, TNF-α and IFN-γ in the tuberculous group were significantly higher than in the inflammatory group. 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 0.702, 0.897, 0.927, and 0.987, respectively for IL-8, TNF α , ADA, and IFN γ. The sensitivity and specificity with IFN γ were 93% and 100%,respectively, which were superior to those for ADA(sensitivity: 80% and specificity:91%), TNF- α (sensitivity: 86% and specificity:91%) and IL-8 (sensitivity: 46% and specificity: 72%) . Conclusions: Levels of the pleural fluid cytokines examined in this study were higher in the tuberculous than in the two other groups. Our data suggested that IFN- γ being shown to be especially very reliable, is a non -invasive and useful marker for diagnosis of tuberculous pleurisy, with clear advantages over ADA.

RIASSUNTO

Lo scopo di questo lavoro era di verificare la validità della misura dell'interferone gamma (IFN-γ) come un marcatore diagnostico per la pleurite tubercolare. In questo studio le concentrazioni nel liquido pleurico della interleuchina-8 (IL-8), del fattore di necrosi tumorale-α (TNF-α), e dell' interferone-γ (FN-γ) sono state pertanto confrontate con quelle della adenosina deaminasi (ADA), in patologie infiammatoria, maligna e tubercolare, al fine di determinare il loro valore diagnostico e di verificare la loro utilità nella diagnosi differenziale di pleurite tubercolare. Lo studio includeva 11 pazienti con patologia infiammatoria, 13 pazienti con patologia maligna e 15 pazienti con pleurite tubercolare, comparabili per età e sesso. Nel liquido pleurico le concentrazioni di ADA, IL-8, TNF-α, e IFN-γ erano significativamente più elevate nel gruppo tubercolare che nel gruppo della patologia maligna. Inoltre le concentrazioni nel liquido pleurico di ADA, TNF-α e IFN-γ erano più elevate nella patologia tubercolare rispetto alla patologia infiammatoria. L'analisi delle curve "receiver operating characteristic" (ROC), ai fini di valutare l'utilità dei differenti parametri, mostrava valori dell'area sotto la curva (AUC) di: 0,702 (IL-8); 0,897 (TNF-α); 0,927 (ADA); 0,987 (IFN-γ). La sensibilità e la specificità di IFN-γ (93 % e 100 %) erano superiori a quelli di ADA (80% e 91%), di TNF-α (86 % e 91 %) e di IL-8 (46 % e 72 %). In conclusione, nel liquido pleurico le concentrazioni delle citochine esaminate in questo studio erano più alte nella tubercolosi che negli altri due gruppi. I nostri dati suggeriscono che la determinazione dell'IFN-γ appresenta un marcatore non invasivo e utile per la diagnosi di pleurite tubercolare, dimostrando chiari vantaggi nei confronti di ADA essendosi dimostrato molt valido.

INTRODUCTION

Tuberculosis probably stands as the most important infectious disease in humans. An estimated 1.7 billion people are infected with the Mycobacterium tuberculosis. Ten millions new cases of tuberculosis occur worldwide every year with about 3 million deaths annually. Ninety five percent (95%) of new cases of tuberculosis occur in the developing countries. In 1998, it became a social issue because an elevation in the prevalence was noted after a long period of decrease(1). Among the extrapulmonary presentations, tuberculous pleurisy is second in frequency after tuberculous lymphadenitis (29%)(2). Conventional methods for the diagnosis of tuberculous pleurisy have proven inefficient. Direct examination of pleural fluid and Ziehl-Neelsen staining requires bacillar concentrations of 10,000/mL and, therefore, has a low sensitivity (0-1%)(3,4). Although a culture is more sensitive (11 to 50%)(5,6), it requires 2 to 6 weeks to grow Mycobacterium tuberculosis and a minimum of 10 to 100 viable bacilli. The

sensitivity of pleural biopsy specimens is reported higher whether by culture (39 to 79%)(4,5) or histologic evaluation (71 to 80%)(3,4). However, this procedure requires greater expertise, is more invasive, and is subject to sampling error(7).

Although tuberculous pleural effusion may resolve over a period of several months without treatment, a failure to diagnose and treat tuberculous pleurisy can result in progressive disease and the involvement of other organs in as many as 65% of patients(8). However, treatment based on clinical suspicion rather than on microbiological diagnosis results in overtreatment, delay in accurate diagnosis, and potentially greater morbidity(7).

For daily clinical activity, however, investigation of markers in pleural fluid is far easier to perform than histological methods. Determination of adenosine deaminase (ADA) activity in pleural fluid, which is due principally to ADA_2 produced by monocytes(9) and is indicative of a local, active, inflammatory response, has been in fact considered as a useful supplemental diagnostic index for tuberculous pleurisy. However, the sensitivity and specificity of this test vary from one laboratory to another with false positive and false negative results (10,11).

Recently, it has been revealed that various cytokines such as interferon gamma (IFN)-γ, interleukin-8 (IL-8) and tumour necrosis factor a (TNF-α) are intimately involved in the pathognomonic physiology of tuberculosis(12). These cytokines are thought to play a role in human-cell mediated immune response to microbacterial infection. They enhance macrophage phagocytic capacity and perhaps microbacterial killing(13,14,15).

In this study, IL-8, TNF-α and IFN-γ were therefore compared with ADA in exudative pleural effusion of inflammatory, malignant and tuberculous origin to determine their diagnostic value and whether either or all of them could be helpful in the differential diagnosis of tuberculous pleural effusion. The utility of each marker was evaluated by analysing its sensitivity, specificity and receiver operating characteristic (ROC) curve.

SUBJECTS AND METHODS

Thirty nine patients with exudative pleural effusion were diagnosed and choosen to enter this study. Patients were subjected to thorough history taking and physical examination and they underwent the following investigations: -X ray chest (posteroanterior and lateral views). -Complete blood picture and sedimentation rate. -Tuberculin test. -Sputum examination for mycobacterium tubercle bacilli by Ziehl-Neelsen stained smear . -Aspiration of pleural fluid for measurement of protein, LDH, ADA, bacteriologic examination and cytologic examination. Some of the pleural fluid was frozen to be used later on for determination of TNF- α, IL-8 and IFN- γ . Serum measurement of proteins and LDH were done at the same session of pleural fluid aspiration.

Pleural fluid was designated as exudate according to Light's criteria with LDH in pleural fluid greater than 200 u/L, pleural fluid /serum LDH ratio greater than 0.6 and pleural fluid/serum protein ratio greater than 0.5(16).

-Other investigations were done in some patients when needed to reach the proper diagnosis including : liver and kidney function tests , abdominal ultrasonography, computerized chest tomography, fiberoptic bronchoscopy and closed pleural biopsy.

Accordingly patients were divided into three groups:

Group 1: included 11 patients with inflammatory pleural effusion (parapneumonic in 8 cases and pyothorax in 3 cases). They were 7 males and 4 females with mean age of 49.36 years.

Group 2: included 13 patients with malignant pleural effusion (lung cancer in 10 cases and metastatic effusion in 3 cases). They were 8 males and 5 females with mean age of 52.31 years.

Group 3: included 15 patients with tuberculous pleural effusion. They were 9 males and 6 females with mean age of 48.13 years.

The cytokines IL-8, TNF- α and IFN- γ were estimated in the frozen pleural sample and compared between the different groups. Serum estimation of these cytokines were also done. **Pleural fluid sampling:** samples of pleural effusion were obtained by thoracocentesis before patients were given any treatment . After centrifugation at 3000 rpm for 5 minutes , the supernatant was frozen at -80°C.

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. ADA activity was determined in 1ml pleural fluid using the colorimetric method described by Giusti and Galanti(17) .

Determination Of Cytokines:

-IL-8 concentration was estimated by an immunoenzymometric assay for the quantitative measurement of human IL-8 in biological fluids (Bio Source Europe S.A, Belgium) (18).

-TNF-α concentration was estimated by an immunoenzymometric assay for the quantitative measurement of human TNF- α in biological fluids (Bio Source Europe S.A, Belgium) (19) .

-IFN- γ concentration was estimated by an immunoenzymometric assay for the quantitative measurement of human IFN- γ in biological fluids (Bio Source Europe S.A, Belgium) (20) .

STATISTICAL ANALYSIS

The data were analysed using the Mann-Whitney method for comparison of mean $\pm$ standard error. Pearson's correlation coefficients ''r'' were used to describe correlations between levels of measured parameters. 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) (21).

RESULTS

Results of the study are shown in tables 1, 2 and figures (1-6)

There were no difference in age and gender distribution among the different groups.

The mean level of pleural fluid ADA in the tuberculous group was 72.267±7.61 IU L^{-1}. This was significantly higher than in the other groups, with values of 36.36±6.34 IU L^{-1} ($p<0.01$) for the inflammatory, and 20.23±2.29 IU L^{-1} ($p<0.01$) for the malignant group(fig 1). The mean level of pleural fluid IL-8 in the tuberculous group was (2216±730.96 pg ml^{-1}), significantly higher than that (365.69±77.38 pg ml^{-1}) of the malignant group ($p<0.05$). It was 769.55±182.71 pg ml^{-1} in the inflammatory group (fig 2). TNF-α level was 25.93±3.5 pg ml^{-1} in the tuberculous group which is significantly higher than in the two other groups, with values of 9.65±1.68 pg ml^{-1} ($p<0.05$) for the inflammatory group and 7.03±0.7 pg ml^{-1} ($p<0.01$) for the malignant group (fig 3). The difference in values for IFN-γ was even more pronounced. The level in the tuberculous group was 56.13±8.44 IU ml^{-1} , and only 0.99±0.17 IU ml^{-1} in the inflammatory, and 0.56±0.09 IU ml^{-1} in the malignant group which show highly significant difference between both inflammatory and malignant groups as compared to tuberculous group ($p<0.01$ for both) (fig 4).

Table 1
Age distribution and sex incidence

	Age (years)				Sex incidence	
	No.	Range	Mean	SD±	Male	Female
Group 1 (inflammatory)	11	25-62	49.36	9.1	7	4
Group 2 (Malignant)	13	41-72	52.31	9.01	8	5
Group 3 (tuberculous)	15	20-65	48.13	12	9	6

Table 2
Sensitivity and specificity on diagnosis of tuberculous pleural liquid

	IL-8 (pg ml^{-1})	TNFα (pg ml-1)	ADA (IUL^{-1})	IFNψ ($IUml^{-1}$)
Cut-off value	875	13	35	3.1
Sensitivity (%)	46	86	80	93
Specificity (%)	72	91	91	100

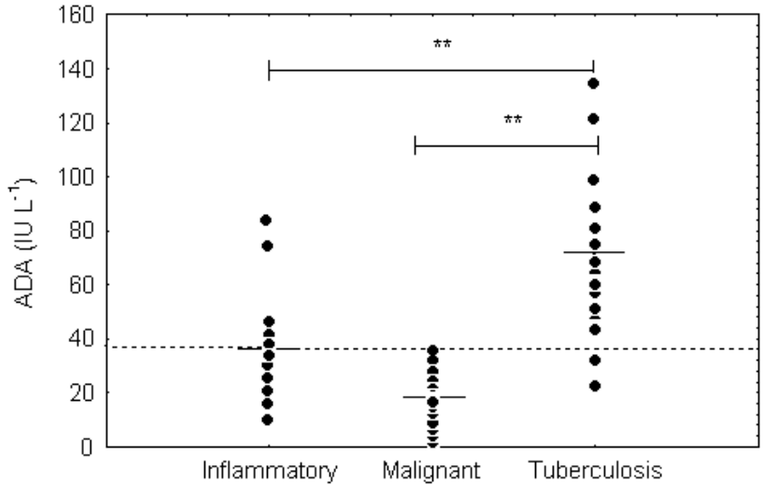

Figure 1
*ADA in pleural effusions. Mean (—), cut-off (-----), ** p < 0.01*

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, and the positive rate (sensitivity) on the vertical axis (fig 5). The AUC value for each was calculated, and the diagnostic accuracy was compared. IL-8 and TNF-α showed intermediate accuracies of 0.702 and 0.897 respectively. The results for ADA and IFN- γ were both high at 0.927 and 0.987 re-

spectively.

The cut-off value 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 IL-8, TNF -α, ADA, IFN γ they were 875 pg ml^{-1},13 pg ml^{-1}, 35 IU L^{-1} , 3.1 IU ml^{-1} respectively (table 2) . The sensitivity and specificity with IFN γ were 93% and 100%,respectively, which were superior to those for ADA(sensitivity: 80% and specificity:91%), TNF- α (sensitivity: 86% and specificity:91%) and IL-8 (sensitivity: 46% and specificity: 72%) (table 2).

Adenosine deaminase activity was significantly correlated with IFN-γ level(r =0.58 P<0.05) (Fig 6)

The concentration of different cytokines (IL-8, TNF- α and IFN- γ) in serum were not significantly different among the three groups and their levels were very low in comparison to pleural effusion levels (data not shown).

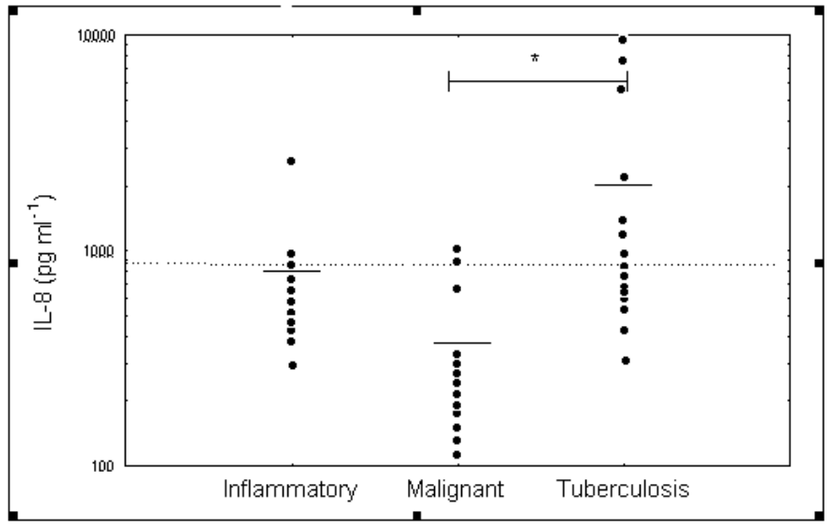

Figure 2
*IL-8 in pleural effusions. Mean (—), cut-off (-----), * p < 0.05*

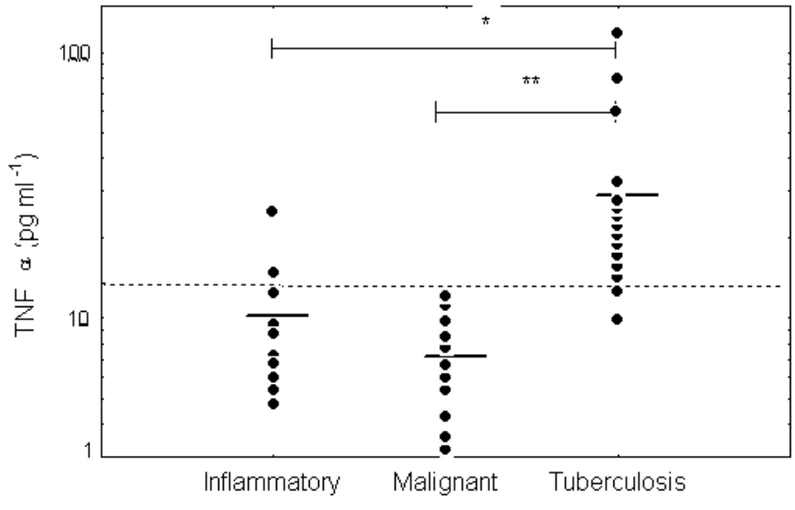

Figure 3
*TNF in pleural effusions. Mean (—), cut-off (-----), * p < 0.05, ** p < 0.01*

DISCUSSION

Although tuberculosis, malignancy and bacterial infection are the major causes of exudative pleural effusion, it is not uncommon for the presence of pleural effusion to present a diagnostic dilemma. The most reliable method for the diagnosis of tuberculous pleurisy is identification of Mycobacterium tuberculosis in pleural effusion(22). The positive rate with smear testing for tubercle bacilli in pleural fluid was less than 10%, that with culturing ranged from 20% to 30%(23). Accordingly when the test is negative , the diagnosis should be established histopathologically with a pleural biopsy by Cope's needle or thoracoscopy. However, the diagnostic rate with pleural biopsy ranged from 50% to 80% (23). It is obvious that the availability of a reliable, yet simple, diagnostic test that replaces invasive histopathological examination would add a great advantage to the establishment of a definite diagnosis(12).

Recently, making a diagnosis at an early stage has been greatly facilitated by detection of tubercle bacilli in pleural fluid with the PCR method. However, the reported range for positive rates is as wide as 12-100% (24). Pleural fluid ADA has long been used as a marker for tuberculous pleurisy , with

sensitivities ranging from 93% to 100%, and specificities from 76% to 100%(11). 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 immunity (23). The results of the present study also showed the ADA levels in the tuberculous group to be significantly higher than in the malignant and inflammatory groups. As described previously , ADA seems to be a useful marker of tuberculous pleurisy.

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, TNF- α and IFN- γ are produced (25,26).

IL-8, which stimulates T cells, is known to play an important role in granulomatous process (27). Production of IL-8 by monocytes after phagocytizing tubercle bacilli has been described (28), but some researchers reported high levels in pleural fluid associated with either pyothorax or pneumonia, correlating with neutrophil counts and extent of myeloperoxidase activity (29,30). In one report IL-8 elevation was found to be greater with pyothorax or pneumonia than with tuberculosis or malignancy (31). In our study IL-8 was particularly elevated in the tuberculous group & this was significantly high when compared to the malignant group but not the inflammatory group. 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.

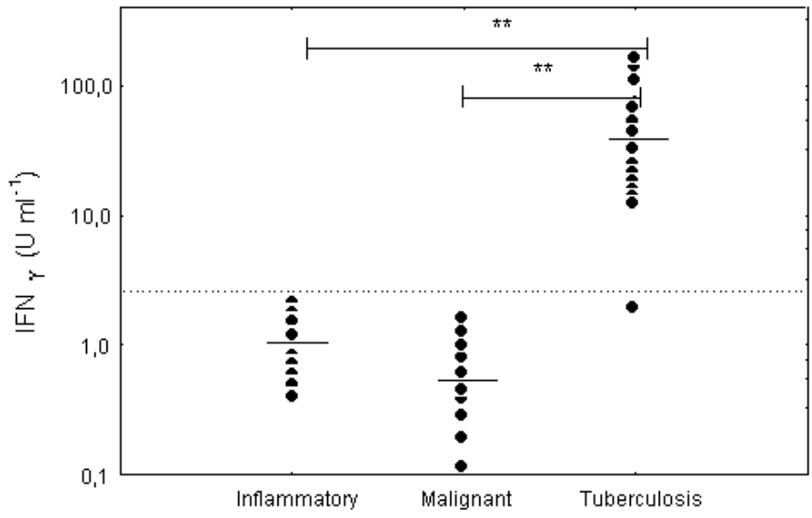

Figure 4
*IFNγ (IU ml^{-1}) in pleural effusions. Mean (—), cut-off (-----), ** p < 0.01*

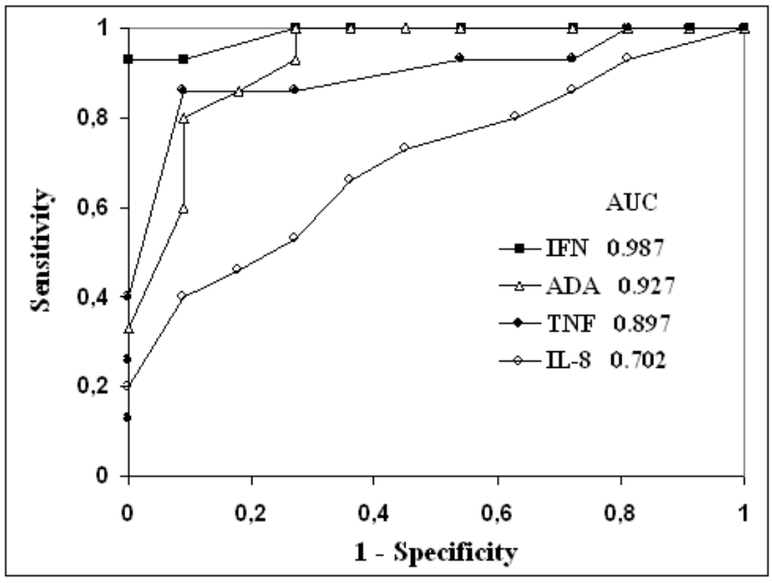

Figure 5
ROC curves for diagnosis of tuberculosis. AUC: area under the curve. IFNγ showed a maximal area (0.987). ADA, TNFα and IL-8 recorded 0.927, 0.897 and 0.702, respectively

Higher TNF- α levels in tuberculous as compared to the other groups were found in this study as in other studies (32,33). TNF- α is considered necessary for producing granulomas and removing rod-shaped bacteria in inflammatory lesions, and it is also regarded as an inducer of IFN- γ. In rheumatic pleural effusion , TNF- α increases in a similar manner as in the tuberculous case. However down-regulation of IFN- γ production occurs in rheumatic pleural liquid (34). A number of researchers have reported that distinction of tuberculous from rheumatic or inflammatory pleural effusion cannot be made with TNF- α (34).

The T lymphocyte re-

sponse plays an important role in the pathogenesis of clinical manifestations and control of tuberculosis. Tuberculous pleural effusions have an increased percentage and an increased absolute number of T-lymphocytes compared with peripheral blood. Other types of effusions also have increased percentages of T-lymphocytes but the absolute number of lymphocytes is not elevated (35).

Pleural infections by Mycobacterium tuberculosis are accompanied by a lymphocytic infiltrate and formation of an exudates rich in T-lymphocytes, predominantly T4 lymphocytes. In vitro stimulation with purified protein derivative (PPD) leads to a proliferative response (36). Shiratsuchi and Tsuyuguchi (37) proved that PPD-induced proliferating lymphocytes mainly belonged to the T4 subset. The stimulation of T-lymphocytes also is accompanied by the production of IFN- γ (38). Some authors proved that different T-cell subsets could produce IFN- γ, with results depending on the technique, stimulus and lymphocytes used (39). Shimokata et al (40) showed that T4 lymphocytes are responsible for in vitro production of IFN-γ when the tuberculous pleural lymphocytes are stimulated by PPD.

The elevated IFN-γ levels found in tuberculous pleural fluids might be the equivalent in vivo of the production observed in vitro after PPD stimulation . IFN-γ detected in pleural fluid may be the result of the in situ stimulation of T4 lymphocytes by tuberculous antigens. IFN-γ is known to activate macrophages, increasing their bactericidal capacity against Mycobacterium tuberculosis (41), and when they are treated with CD4 monoclonal antibodies and complement, IFN-γ levels decreases (34). Thus 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 IFN-γ (34). A number of reports have demonstrated that IFN-γ levels in patients with tuberculous pleurisy are high, with sensitivities and specificities ranging from 90% to 100% (34,42:47). Valdes et al. (11) conducted research on pleural fluid samples obtained from 145 patients and reported two with small volumes out of 35 tuberculous cases to be false-negative, while nine out of 110 non-tuberculous cases were false-positives (one ; parapneumonic pleural effusion, three; pulmonary embolism, three; lymphoma, one; lymphocytic leukemia, one; neuroblastoma) (28). In our study there were no false-positives with the use of IFN- γ, and only one case with a small volume of pleural fluid presented as a false-negative. These findings provide strong support for the conclusion that IFN-γ is a reliable marker of tuberculous pleurisy.

Our finding of high levels of these cytokines in tuberculous pleural effusion together with the finding of very low levels in the serum , may support the suggestion that these cytokines are produced locally by inflammatory cells(12,28,41) .

ROC curves can profile sensitivity and specificity of markers, and are regarded as useful for analyzing and/or comparing diagnostic accuracy (48) . While ADA was here found to have good values for both parameters , IFN-γ was superior as a marker for tuberculous pleurisy. All of the three cases with false -positive ADA findings were cases with pyothorax, and their IFN-γ levels were all lower than the cut-off value. There were two cases showing false-negatives for ADA , but they had high IFN-γ levels. In these two cases , pleural effusion was aspirated after one month of onset. ADA levels in tuberculous pleurisy may decrease long after onset, but, even if this is also the case for IFN-γ, elevation above the cut-off value seems to be maintained.

Although the rises in ADA and IFN-γ levels in tuberculous pleural effusion have different origins (infected macrophages in the case of ADA and sensitized CD4+ cells in that of IFN-γ), ''in this study and another study (49) although not in certain others (50)'' ADA and IFN-γ were correlated.

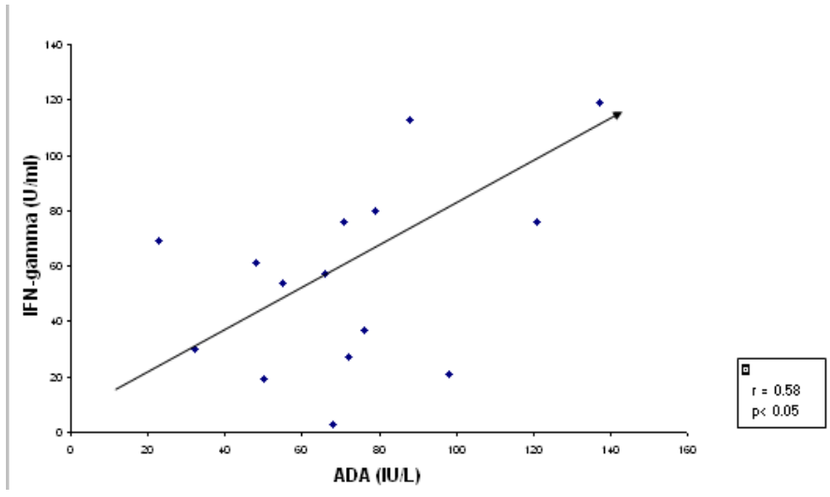

Figure 6
Correlation between ADA and IFN-gamma levels in tuberculous pleural effusion

As we have found gamma interferon to be a highly specific finding in tuberculous effusions, an assay for IFN-γ may prove to be useful as a screening test for tuberculous pleurisy. Nevertheless, the measurement of IFN-γ is an expensive technique when compared with adenosine deaminase determinations , an other excellent method for rapid screening of tuberculous effusions. The cost of a test becomes an important consideration when one is dealing with a disease with a higher incidence in less developed countries.

In conclusion , Levels of the pleural fluid cytokines

examined in this study were higher in the tuberculous than in the two other groups. Our data suggested that IFN-γ being shown to be especially very reliable, is a non-invasive and useful marker for the diagnosis of tuberculous pleurisy, with clear advantages over ADA.

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