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Tuberculous Effusion: ADA Activity Correlates with CD4+ Cell Numbers in the Fluid and the Pleura

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Key Words
T cells · Adenosine deaminase · Macrophages · Tuberculosis · Pleural effusion

Abstract
Background: Adenosine deaminase (ADA) is a commonly used marker in the diagnosis of tuberculous effusion and there is evidence that its production is linked to T cells and monocytes. Data on the correlation between ADA and T cells or macrophages in tuberculous effusions are conflicting. Furthermore, no studies have examined a possible correlation between pleural tissue infiltration and ADA. ***Objectives:*** We undertook this study to examine cell subsets in the fluid and the pleura in tuberculous effusion and their correlation to ADA. The use of cell subsets as a marker in the differential diagnosis was also examined. ***Methods:*** Pleural fluid from 36 patients with tuberculous and 34 patients with malignant effusion as well as pleural tissue biopsies from 16 patients with tuberculous pleurisy were examined. The APAAP and the avidin-biotin complex immunocytochemical methods were used to examine CD4+ T cells and macrophages (CD68+), while ADA activity was measured by the Giusti colorimetric method. ***Results:*** Our results showed that, in pleural fluid, CD4+ cells and ADA were significantly higher in tuberculous compared to malignant effusion ($p < 0.001$ for all measurements). In pleural tissue biopsies, macrophages were the predominant cells but CD4+ T cells were also abundant. A significant correlation was found between ADA and CD4+ numbers in pleural fluid and tissue ($r = 0.45$, $p < 0.01$; $r = 0.75$, $p < 0.001$, respectively). ADA had high sensitivity and specificity for differential diagnosis while cell subsets did not. ***Conclusions:*** These results indicate that ADA activity correlates to CD4+ T cell infiltration in the pleura and the fluid. Moreover, ADA but no cell subsets may be used as markers of tuberculous effusion.

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Introduction

Adenosine deaminase (ADA) is a commonly used marker in the diagnosis of tuberculous pleural effusions and its value has been reported in a number of studies [1–9]. Enzyme activity is found in all cells with the highest activity in lymphocytes and monocytes [10]. Both types of cells characterize tuberculous reactions and their presence has been shown in the lung parenchyma [11, 12], the

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tuberculin skin reaction [13] and in pleural fluid [14–16]. However, similar cell counts are also reported in malignant effusions where no significantly high ADA activity is usually detected [3, 14, 15, 17]. Furthermore, while some studies show a positive correlation between T cells and ADA in tuberculous effusion, other studies fail to demonstrate such a correlation [18–20]. Moreover, it is possible that ADA levels are linked to pleural tissue T cell and macrophage infiltration and this has not been examined.

The aim of this study was to examine the presence of macrophages and CD4+ T cell subsets in the pleura and the fluid in tuberculous effusion and their possible correlation with ADA levels in the fluid. Data on this correlation might provide us with a better understanding of the source of ADA production in vivo. Findings were compared to those in malignant effusion. The use of cell subsets as a marker in the differential diagnosis was also examined.

Methods

Patients

Pleural fluid from 70 patients was examined. Thirty-six patients had tuberculous pleurisy (20 men, mean age 31, range 20–87), the diagnosis of which was confirmed by pleural biopsy (30 cases), positive pleural fluid culture (4 cases) or polymerase chain reaction (PCR; 2 cases who also responded well to antituberculous treatment). The histological diagnosis of the pleural disease was assessed by light microscopy on hematoxylin and eosin slides, finding generally small, caseating or noncaseating granulomas in the pleura usually against a background of lymphocytes. PCR was performed using the AMPLICOR *Mycobacterium tuberculosis* PCR test (Roche Diagnostic Systems, Branchburg, N.J., USA) [21]. Testing was made according to the manufacturer's recommendations. Two pleural fluid samples of 100 μl were examined for each patient and both tested positive. Furthermore, both patients had pleural fluid ADA titers >70 IU/l. All patients had a Mantoux reaction >10 mm and all patients were seronegative for HIV. Pleural tissue from 16 patients was additionally examined using immunohistochemistry.

Thirty-four patients had malignant effusion confirmed at cytology (27 men, mean age 49, range 27–68; 18 adenocarcinomas, 9 squamous cell carcinomas, 6 small cell carcinomas, 1 mesothelioma).

Immunocytochemistry and ADA Measurement

Pleural fluid was aspirated prior to initiation of treatment. A small sample was used for evaluation of total cell numbers and viability and to perform differential counts. Two milliliters were used for immunocytochemistry and within 20 min they were cytocentrifuged on slides, using 150-μl aliquots and a cytospin 3 (Shandon, UK). Slides were air dried for 1 h, wrapped in pairs back to back in aluminum foil and stored in –20 °C prior to immunostaining.

Slides were allowed to defrost in their foil covering. They were then fixed in equal parts of acetone-methanol for 5 min and placed in phosphate-buffered saline. Monoclonal antibody staining was detected by a modification of the alkaline phosphatase-antialkaline phosphatase method described by Mason and Sammons [22]. Twenty percent normal human serum was used to prevent nonspecific binding of the second and third layer antibodies. System and specificity controls were included in each staining run, using human tonsil obtained at routine tonsillectomy operations, and a mouse IgG2a myeloma protein as a negative control. The monoclonal antibodies used were anti-CD4 (T helper cells; Becton Dickinson, Cowley, Oxford, UK) and anti-CD68 (macrophages; Dako, High Wycombe, UK). The remaining pleural fluid sample was centrifuged at 3,000 rpm for 15 min to pellet the cellular elements. Samples of the supernatant were frozen at –20° until assayed. ADA activity was determined by the Giusti colorimetric method [23].

In pleural tissue biopsies immunohistochemical staining was carried out using the monoclonal antibodies CD45RO clone OPD4 (anti-CD4) and clone KP1 (antimacrophages; Dako) in sequential 5-μm sections of formalin-fixed, paraffin-embedded tissue blocks from the biopsy specimens. Immunohistochemistry was performed according to a modified method of Hsu et al. [24] using as detection system the avidin-biotin alkaline phosphatase (Vectastain ABC kit). Briefly, the sections from formalin-fixed, paraffin-embedded tissue blocks were mounted on slides coated with Vectabond (Vector Laboratories), deparaffinized in xylene, rehydrated and rinsed in TBS buffer. The sections for macrophage detection were immersed in 10 m*M* antigen retrieval citrate buffer solution and irradiated in a microwave oven for 5 min (3 times) at 600 W. The sections were kept for 30 min at room temperature, then washed in phosphate-buffered saline. Next all the sections were incubated with the blocking serum for 20 min and without rinsing were incubated with the specific primary antibody overnight (18 h) at 6 °C. The avidin-biotin reagents were incubated for 30–40 min each at room temperature and were then developed with Fast Red salt and were counterstained with Mayer's hematoxylin.

Slides were coded and cells counted in a blinded fashion using a BH2 microscope (Olympus, Japan) and an eyepiece graticule at a magnification of 200. The numbers of positively stained cells were counted and expressed per high-power field. A minimum of two slides were stained and counted for each antibody from each patient and the mean value was calculated. The coefficient of variation for three repeat cell counts by the same observer for each antibody was <5% and the interobserver variation was <9%.

Data Analysis

Statistical analysis was performed using statistical software (Sigma Stat 3.0, Jandel Scientific, San Raphael, Calif., USA). Between-group analyses comparing cell counts were performed using the Mann-Whitney rank sum test. Correlations between cell counts and ADA were analyzed using Spearman's rank method. Sensitivity and specificity were assessed using the ROC analysis. p values ≤ 0.05 were considered significant.

Results

Differential cell counts of the pleural fluid showed lymphocytic predominance in both tuberculous and malignant effusions. Immunocytochemistry showed that this predominance was mainly due to CD4+ T cells. However, the numbers of CD4+ T cells were significantly higher in

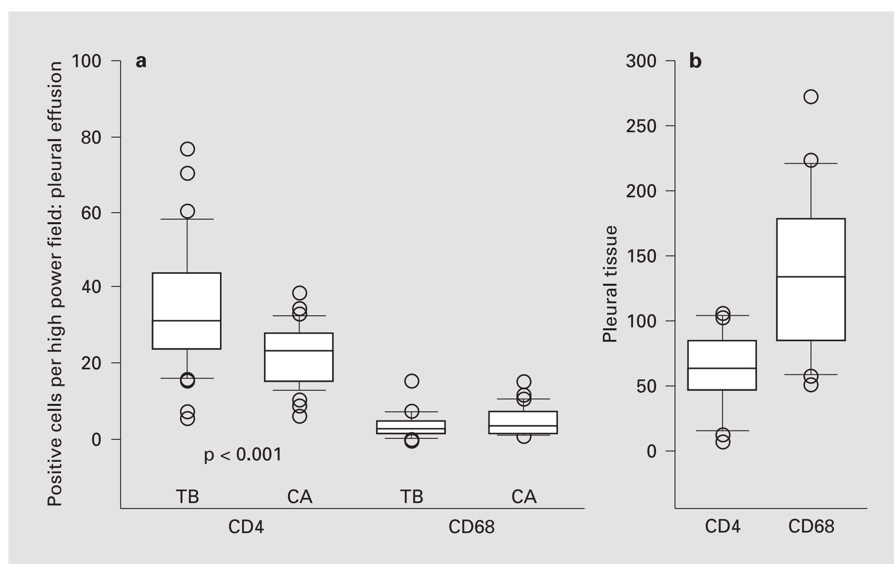

Fig. 1. CD4+ T cell subsets and macrophages (CD68+) in tuberculous (TB) and malignant (CA) pleural effusion (**a**) and pleural tissue (**b**). Values expressed as cells per high power field (×200). Boxes represent median values and 95% CI. Lower and upper bars represent 10–90 centiles.

Table 1. Cell counts (%) and ADA value (IU/l) in pleural fluid

	Tuberculosis	Malignancy	p value
CD4	30.7 (15.6–58.3)	23.1 (12.7–32.2)	<0.001
CD68	2.3 (0.3–7.6)	3.3 (0.88–10.5)	<0.01
ADA	94 (69–111)	28 (19.8–33.3)	<0.001

Values represent median with 10–90% in parentheses.

Table 2. Sensitivity and specificity (%) of ADA and all cell types as diagnostic markers

	ADA	CD4	CD68
Sensitivity	97.3	75.6	51.3
Specificity	97.1	77.1	75

tuberculous than in malignant effusion ($p < 0.001$). Macrophage numbers were low in both effusions and no differences were noted between the two groups (table 1, fig. 1).

In pleural tissue, cells were abundant in the granulomas but some were dispersed throughout the tissue. Macrophages were the predominant cells infiltrating the pleura and the median value was 133.5 (58.4–220.3) per field. The median value of CD4+ cells was 63.3 (15.1–102.8). Most of the CD4+ cells were confined in the granulomas, but quite a few were dispersed throughout the tissue and also formed small aggregates (fig. 1, 2).

ADA values were significantly and markedly higher in tuberculous effusion and a significant correlation was found between ADA and CD4+ T cells in both pleural fluid ($r = 0.45$, $p < 0.01$) and tissue ($r = 0.75$, $p < 0.001$) (fig. 3). No correlation was found between macrophage numbers and ADA in the pleura or the fluid.

Considering ADA as an aid to a differential diagnosis and using either 50 or 47 IU/l as cutoff points, which are the referenced values [25, 26], both specificity and sensitivity for ADA are high, 97.3 and 97.1%, respectively. In contrast, using the ideal cutoff point according to the ROC analysis, for each cell subset, specificity and sensitivity remain low (table 2). Using ADA and cell counts simultaneously does not further enhance ADA sensitivity or specificity as a diagnostic marker.

Discussion

This study shows that tuberculous and malignant pleural effusions are both characterized by high CD4+ T cell numbers, although tuberculous effusions have significantly higher CD4+ numbers. Macrophages form a small percentage of cells in both types of effusion. By contrast, macrophages are the predominant cells infiltrating the pleura in tuberculous effusion while CD4+ cells are also numer-

ous. The most striking difference between tuberculous and malignant effusion is found in ADA activity that is significantly and markedly increased in tuberculosis. A significant correlation exists between ADA and CD4+ T cells in pleural fluid and tissue

High T cell numbers and ADA activity in tuberculous effusion are reported in the literature [26] and this is in accordance with our findings. However, results on the correlation between ADA and T cells are conflicting [18, 19]. In an older study [18], no correlation was found between ADA and T cells, while in a more recent one [19] a good correlation was reported. We have examined a fairly large number of patients in whom a significant correlation exists between ADA activity and numbers of CD4+ lymphocytes. The difference in ADA activity between tuberculous and malignant patients may not be explained by differences in cell numbers alone. There are a number of studies which show that T cells from tuberculous pleural effusions are memory cells [27]. These T cells are more responsive to priming by macrophages derived from the same effusions and they secrete more cytokines than blood T cells from the same patients or T cells from healthy donors [28, 29]. Urdaneta et al. [30] showed that CD4+CD45RO+ T cells were markedly higher in localized disease compared to diffuse infiltrates, indicating a role for these cell subsets in successful response against tuberculosis. Furthermore, in a study of the tuberculin reaction, Tsicopoulos et al. [31] showed that in contrast to allergen-induced late phase skin reaction, tuberculin induced delayed-type hypersensitivity reaction is characterized by Th1 CD4+ cells which secrete cytokines such as

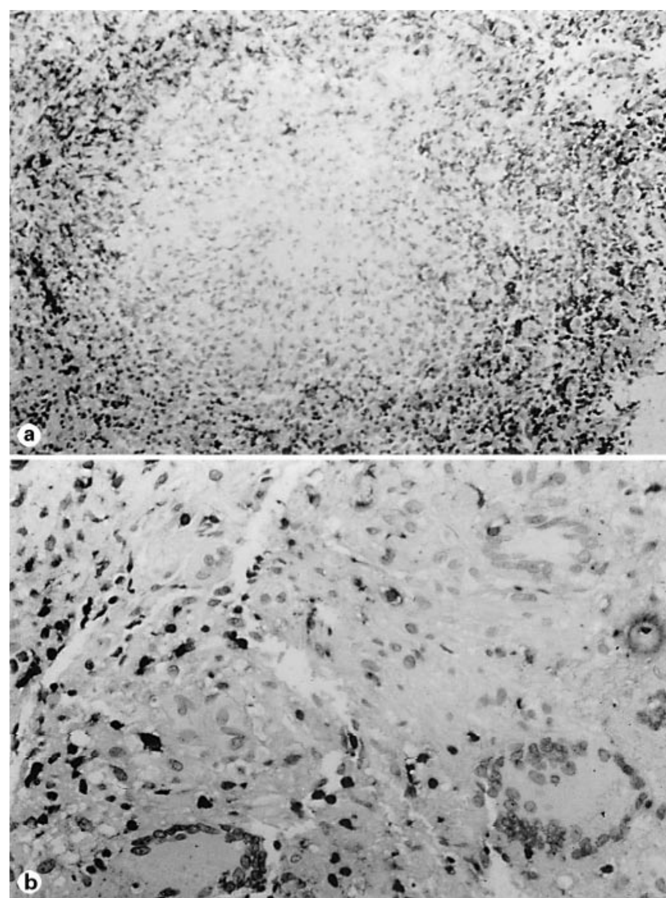

Fig. 2. Photomicrographs of pleural tissue biopsies. **a** Granuloma at low magnification (objective lens ×16) with CD4+ cells stained black. **b** Pleural tissue biopsy at higher magnification (objective lens ×40) with CD4+ cells stained black.

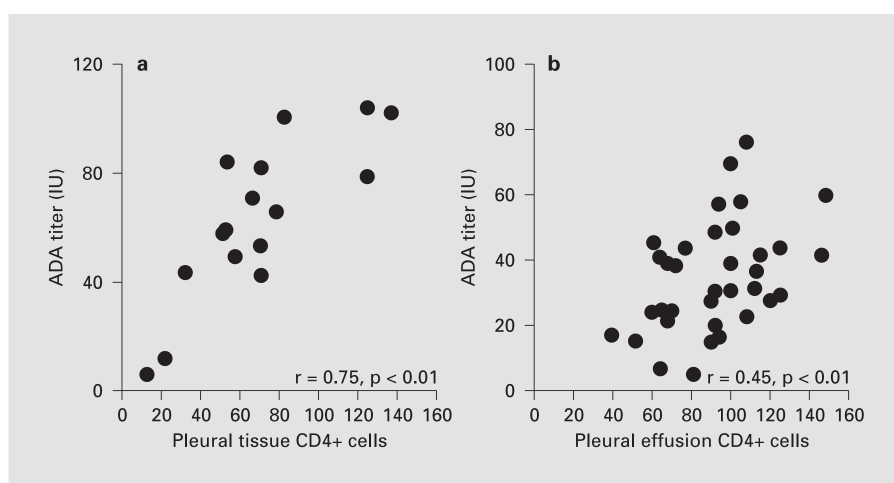

Fig. 3. Correlation graphs between ADA and CD4+ cells in tuberculous pleural tissue (**a**) and pleural fluid (**b**).

IFN-γ and TNF. As far as we know, the Th1, Th2, or Th0 phenotypes have not been extensively investigated in cancer. Differences in ADA activity between tuberculosis and malignancy may be due to differences in TH phenotypes or the presence of memory CD4+ T cells in tuberculosis and further studies should probably be undertaken.

One of the ADA isoenzymes, ADA-2, is mainly responsible for ADA activity in tuberculosis [9, 32] and this enzyme probably has its origin in monocytes and macrophages [33]. We have measured total ADA activity and not ADA-2, which correlates well with total ADA activity [5] and which, according to some studies, does not seem to further improve sensitivity or specificity of the method [9, 34]. On the other hand, there are reports showing that ADA-2 and more specifically the ADA-1/ADA total ratio in pleural fluid can increase specificity to 98.6% [35]. Gakis et al. [33] have suggested that stimulation of macrophages by the phagocytosis of microorganisms such as *M. tuberculosis* allows the release of ADA-2, causing the increase of ADA levels in tuberculosis. However, in our study, no correlation was found between total ADA activity and macrophage numbers in the fluid or the pleura.

When considering ADA and cell subsets as an aid to differential diagnosis, ADA showed very high sensitivity and specificity and this finding further confirms data already reported [3, 8, 9, 36, 37] and applied in clinical practice. ADA levels may also be high in some cases of adenocarcinomas, lymphomas, complicated parapneumonic effusions and, as recently reported, in brucellosis [38] but the value of ADA in the diagnosis of tuberculous effusion remains high especially in areas with high tuberculous prevalence. IFN-γ has also been examined as a potential marker of tuberculous effusion and its sensitivity and specificity are comparable to those of ADA [6, 39] but the cost and slowness in obtaining results have limited its routine use [40]. Other potential markers such as TNF [41] and IL-6 [42] were not shown to be equally sensitive or specific. Moreover, our study showed that the use of cell subsets as a diagnostic marker does not help diagnosis, because although there are statistically significant differences between the two groups, these differences are not clinically important as values overlap and therefore sensitivity and specificity are low.

In conclusion, ADA titer but not T cell subsets may be used as markers of tuberculosis. A significant correlation was found between enzyme activity and CD4+ cell numbers in pleural tissue and fluid. It is possible that ADA is mainly produced in the pleural tissue and spilled over into the fluid.

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