Serum and Pleural Adenosine Deaminase*  
Correlation with Lymphocytic Populations

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This study attempts to correlate levels of ADA in tuberculous and neoplastic pleural exudates with the different immunologic cellular expressions that follow these clinical situations. Seventy-three patients with pleural effusion were studied in order to assess ADA activity (pleural and serum); in 25 of them, a study of delayed cellular immunity (pleural and sanguineous) was performed through B, CD3, CD4, and CD8 lymphocytic populations. The activity of ADA was determined, and the study of lymphocytic populations was made through the use of monoclonal antibodies. The data obtained showed the following: levels of ADA were significantly (p<0.0005) higher in the pleural fluid and the serum of tuberculous effusions compared to neoplastic effusions; percentages of CD2 and CD4, T-cells were significantly (p<0.05 and p<0.0005, respectively) greater in tuberculous effusions. The statistical study of the levels of ADA activity and the percentage of CD4, T-cells in pleural exudates produced a significant regression curve (r = 0.612 and p<0.0001) which showed a positive correlation between these two parameters. The pathogenic implications of these results suggest the possibility that ADA could be a new marker of cell-mediated immune activity.

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ADA = adenosine deaminase; ATP = adenosine triphosphate;  
LDH = lactic dehydrogenase; PBS = phosphate-buffered saline;  
PHA = phytohemagglutinin; Con-A = concanavalin A.

Adenosine deaminase catalyzes hydrolytic and irreversible deamination of dioxyadenosine into dioxyinosine and of adenosine into inosine. This enzyme is widely distributed in human tissues in three forms, soluble and interconvertible (small, intermediate, and large, in relation to its molecular weight). The activity of ADA is ten times greater in lymphocytic cells than in erythrocytes and, in relation to the former, is greater in T-lymphocytes than in B-lymphocytes, and varies during T-cell differentiation, with significant increases of its level in immature or undifferentiated states.1

Therefore, some authors1 consider ADA as a marker of cell-mediated immunity, with an increase in its serum level in different diseases. Regarding its main physiologic activity, ADA is related to lymphocytic differentiation and proliferation, showing a significant increase in its values during the myogenic and antigenic response2 of lymphocytes. In addition, Carson and Seegmiller3 found a restriction of lymphocytic blastogenesis following the activation of ADA inhibitors through biologic and nonclarified mechanisms, possibly connected with conversion of dioxyadenosine into dioxy-ATP, which, gathering selectively in lymphocytic cells, would cause its destruction by the inhibition of DNA synthesis. On the other hand, the congenital and genetically determined deficit of this enzyme with its autosomal recessive trait, described in over 30 cases, is usually associated with severe forms of combined immunodeficiency and is responsible for an increase in toxic nucleotides that prevent the differentiation or proliferation (or both) of T-lymphocytes and thus a normal immune function mediated by cells.

The raising of the levels of ADA activity under antigenic stimulation shows the importance of this enzyme in the rapid proliferation of cells in order to prevent the accumulation of toxic metabolites. Therefore, an increase in ADA activity is present in several circumstances: (1) in pleural, pericardic, and peritoneal effusions of a tuberculous nature, significantly when compared with neoplastic and metapneumonic (bacterial and viral) ones;4,7 (2) in pleural effusions that follow rheumatoid arthritis and lymphoproliferative infections and, also, in empyemas, although with levels usually smaller than in tuberculosis;5,8 (3) in tuberculous cerebrospinal fluid, where ADA values are significantly higher than in the normal group and in other neurologic diseases;14 and (4) in peripheral lymphoblasts of patients with acute lymphoid leukemia.

Therefore, the main practical indication of ADA
activity is that the level of ADA leads to the etiologic diagnosis of pleural effusions and, on a small scale, of pericardial and peritoneal ones, thus emphasizing its value in differentiating tuberculosis and neoplasia.1,5,9,14

The interpretation of these phenomena still suggest justified doubts. Indeed, T-lymphocytes predominate both in tuberculous and neoplastic exudates, it being difficult to discover a complete explanation for the existence of such different levels of this enzyme in the two clinical situations; or will it be, as has been pointed out by some authors, that the presence of more immature and reactive T-lymphocytes than in any other disease will be responsible for a response mediated by cells more specific to mycobacterial antigens? In this context, Ocaña et al16 have shown that a parallel correlation does not exist between the number of T-lymphocytes in tuberculous effusions and their levels of ADA activity.

Thus, in an attempt to help explain these doubts, through the study of the lymphocytic populations and their correlation with levels of ADA, we tried to determine the eventual existence of different immunologic expressions in exudates or in the peripheral blood (or both) of patients with tuberculous and neoplastic pleural effusions, suggesting different pathogenic mechanisms in these two clinical situations.

MATERIALS AND METHODS

Pleural Exudates

For the analysis of ADA in pleural exudates, 73 patients were studied, 50 men and 23 women, with an average age of 53.3 ± 20.0 years (range, 11 to 83 years). The differential diagnosis between the two clinical situations studied (tuberculous and neoplastic) was based, besides other laboratory tests (protein, amylase, LDH, and glucose levels, pH values, etc.), on the presence of direct examinations and positive cultures of Mycobacterium tuberculosis, on the histopathology of pleural biopsies, and on a search for malignant cells in the pleural fluid.

This method allowed the authors to form two groups: (1) 35 patients with tuberculous pleural effusions, with an average age of 37.6 ± 2.7 years; and (2) 38 patients with neoplastic pleural effusions, with an average age of 61.1 ± 12.5 years.

The ADA activity was evaluated in these 73 exudates, and in 25 of them (11 tuberculous and 14 neoplastic), the cellular immunity was evaluated through the study of B-lymphocytic and T-lymphocytic populations and of CD4 and CD8 subpopulations, with the determination of CD4/CD8 ratios.

Peripheral Blood

The activity of ADA was also studied in the peripheral blood of 36 patients (15 tuberculous and 21 neoplastic) and in a control group of 110 normal volunteers; in 25 of them (11 tuberculous and 14 neoplastic), cellular immunity was studied according to the patterns used in pleural fluid examination (B-cell and T-cell populations; CD4 and CD8 subpopulations; ratios of CD4/CD8).

The study of ADA activity was made using the colorimetric technique of Galanti et al16 and Giusti.16 One international unit of ADA represents the enzymatic activity that catalyzes a molecule of substrate in standardized conditions of pH and temperature.

Lymphocytic populations were counted with monoclonal antibodies as follows: mononuclear cells of pleural fluid or of blood were isolated by centrifugation in Ficoll-Hypaque gradient, washed in PBS, and suspended in RPMI 1640 at a concentration of 10 x 10^6 cells per milliliter. As monoclonal antibodies, we used those of anti-human R: CD4 (T4); CD8 (T8); CD16 (T6); and CD16 (B).

Then, 5μl of an adequate dilution of monoclonal antibody was added to 50μl of cellular suspension (<5 x 10^6 cells per milliliter) at 4°C for 30 minutes. The cells were then washed three times with PBS; and then, 50μl of an adequate solution of fluorescent-
RESULTS

Conjugated immunoglobulin from rabbit anti-rat was added at 4°C for 30 minutes. Finally, the cells were washed three times and observed with the fluorescent microscope, with at least 300 cells being counted.

In the statistical analysis of the data, we used the Student-Fisher t-test and polynomial regression curve, grade 2.

Activity of ADA

The mean level of ADA activity in the fluids of tuberculous pleural effusions (110.6 ± 35.2 U/L; Fig 1) was significantly (p < 0.0005) higher than in the neoplastic pleural effusions (17.5 ± 8.4 U/L), and it must be pointed out that the lower value in the tuberculous group (47 U/L) was clearly greater than the highest in the neoplastic group (38 U/L).

In serum the differences between these two groups point to the same effect; therefore, the mean value of the tuberculous group (29.8 ± 10.0 U/L) was significantly (p < 0.0005) higher than in the neoplastic group (14.5 ± 4.0 U/L).

The study of the control group allowed us not only to determine the normal serum level of this enzyme (21.3 ± 7.06 U/L), but also to discover that the patients with tuberculous effusions showed levels of ADA activity in serum that were significantly (p < 0.01) higher than normal, and patients with neoplastic pleurisy were lower (p < 0.01).

Lymphocytic Populations

T-cells predominated largely in pleural exudates (with percentages higher than 70 percent) over B-cells, which, on the average, did not reach 10 percent (Fig 2); however, the levels of T-lymphocytes in tuberculous fluids (Fig 2) were significantly (p < 0.05) higher than those in the neoplastic group (86.2 ± 10.0 percent and 73.9 ± 13.3 percent, respectively), while the values of B-lymphocytes were similar (Fig 2) in these groups (9.1 ± 7.5 percent and 9.5 ± 5.1 percent, respectively).

With regard to lymphocytic subpopulations of pleural fluids, the percentage of the CD4 T-cells in tuberculous effusions (65.7 ± 11.7 percent; Fig 2) was significantly (p < 0.0005) higher than in neoplastic effusions (45.6 ± 11.3 percent), but in relation to the CD8 T-cells, the data showed an opposite effect (Fig 2), ie, values significantly (p < 0.005) higher in neoplastic effusions (28.2 ± 7.7 percent) than in tuberculous effusions (18.3 ± 10.0 percent). Thus, the ratio of CD4/CD8 (Fig 2) in tuberculous exudates had a statistically significant higher value (4.3 ± 1.5; p < 0.0005) than in neoplastic effusions (1.7 ± 0.6).

In the peripheral blood of these patients, the data obtained from the study of lymphocytic subpopulations did not show (Fig 3) any statistically significant differences between the two groups studied (tuberculous and neoplastic), although the first showed a higher percentage of CD4 T-cells (44 ± 8.2 percent vs 30.5 ± 11.6 percent), and the second showed a higher percentage of CD8 T-cells (28.9 ± 4.6 percent vs 33.7 ± 9.1 percent), with a CD4/CD8 ratio slightly superior in patients with tuberculous exudates (1.6 ± 0.6 vs 1.3 ± 0.6).

The relationship between ADA levels and the percentages of the CD4 T-cells in all of the exudates studied produced a significant regression curve (r = 0.612 and p < 0.0001) which showed a positive correlation between these two parameters (Fig 4).
DISCUSSION

According to a great number of authors,\textsuperscript{1,4-6,8,10,12,14} the levels of ADA activity found in tuberculous pleural fluids were largely and significantly (p<0.005) higher than those found in neoplastic exudates. In addition to the fact that the lowest value in the tuberculous group was clearly superior to the highest in the neoplastic group, the data obtained allowed us to recognize the great sensitivity (100 percent) and specificity (100 percent) of ADA in the differential diagnosis between these two clinical situations.

Curiously, serum variations of this enzyme, although with mean levels smaller than the pleural ones, pointed to the same significant difference (p<0.0005). Therefore, it seems that also in the blood, there would be present mechanisms responsible for these differences, probably the cellular elements responsible for this enzyme production recirculating between pleura and blood.

Will immaturity and lymphocytic reactive capacity be the unique explanation for the discrepancy of ADA pleural values, with regard to tuberculous or neoplastic etiologies of the effusions? On the other hand, if the increase in the levels of activity of this enzyme confirms ADA reaction/T-cellular response,\textsuperscript{1} then what will be the mechanism that determines the presence

\[ R = 0.78 \]

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{Percentages of lymphocytic subpopulations in blood of two studied groups.}
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\begin{figure}
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\includegraphics[width=\textwidth]{figure4}
\caption{Correlation between ADA levels and percentages of T\textsubscript{4}-cells in pleural effusions.}
\end{figure}
of small quantities of exudates of a malignant nature, in the face of a high T-lymphocytic percentage in the pleural cavity? In fact, and in accordance with other authors, we also discovered a clear percentage of predominance of T-lymphocytes in the pleural fluids of tuberculous and neoplastic etiologies, with verifiable T/B ratios of 9.47 and 7.7, respectively (in peripheral blood, 5.02 and 5.53, respectively).

Nevertheless, the lymphocytic dynamic that follows pleurisy is not totally clarified, especially in regard to the eventual specificity in the attraction of T-lymphocytes or the recirculation of these cells. In fact, a certain independence that some authors recognize in these phenomena at the pleural level could only depend on certain anatomic and physiologic peculiarities of this compartment; among them, it could be pointed out that in pathologic conditions, a small clearance capacity exists (great number of cells and higher lymphokine contents in relation to blood). In our cases of tuberculous pleural effusions, the presence of serum levels of ADA significantly higher than normal, suggesting the recirculation of an important number of activated T-lymphocytes, seems to point in the same direction.

Alternatively, and as has been verified by us over the period of the last two years, the absence of statistically significant differences between the percentages of CD4 and CD8 cells in the bronchoalveolar lavage fluid and in the blood of patients with pulmonary tuberculosis (in relation to the control normal group) seems to indicate that a locally independent cell-mediated immune response would not exist in the pulmonary parenchyma in these circumstances and these clinical situations.

In neoplastic effusions, although there is a high percentage of T-lymphocytes either in vivo or in vitro, a lower or even null capacity of these cells to be stimulated by PHA or by Con-A is seen. On the contrary, in tuberculous pleurisy, T-lymphocytes react intensely to myogenes, specific and nonspecific (PPD, PHA, Con-A, etc).

In fact, the data obtained from the study of the CD4 and the CD8 lymphocytic subpopulations in pleural exudates of tuberculous and neoplastic etiologies showed a significant (p<0.0005) increase in the CD4 T-cells in tuberculous vs neoplastic effusions and also showed in the latter a significant increase (p<0.005) of the CD4 T-cells. Compared to the former, therefore, the ratio of CD4/CD8 T-cells in tuberculous effusions was significantly superior (p<0.0005) to that in neoplastic ones. Thus, in tuberculous pleural exudates, the intense and accelerated blastogenesis that it seems T-cells reach after the mycobacterial antigenic stimulation, and from which ensues a significant increase of the CD4 subpopulation, could explain the synthesis of ADA, whose activity in these processes of lymphocytic proliferation and differentiation appears to be essential.

In fact, recently completed investigations that we hope to publish soon seem to show that the ADA present in the tuberculous and neoplastic pleural exudates is essentially synthesized by the CD4 T-cells. In these circumstances, it was suggestive of the positive and significant relationship between ADA levels and the correspondent percentages of the CD4 T-cells in all of the exudates studied (Fig 4).

These data could explain the discrepancy observed between pleural levels of ADA in exudates of two clinically and immunologically distinct situations, tuberculous and neoplastic pleurisy, whose fluids were also rich in T-cells. In fact, the increase of ADA activity seems to correspond to an increase in the CD4 lymphocytes (tuberculous effusion), while its decline would correlate with a higher percentage of the CD8 lymphocytes and a fall of the CD4 T-cells (neoplastic effusion).

We must discover if the high levels of ADA present in the tuberculous exudates resulted only from the existence, in those fluids, of an increasing number of T4-cells or if, in this clinical situation and integrated in a physiopathologic context, those lymphocytic subpopulations will be stimulated to a greater production of this enzyme, and so contribute to a correct immunologic defense of the body against the tuberculous infection. On the contrary, we also need to discover what would happen in the neoplastic pleural fluids eventually influenced by the decline of the cell-mediated immune protection.

These data seem to suggest to us, as to other authors, that ADA constitutes a new marker of cell-mediated immune activity, since its deficiency, genetically determined, was associated with serious forms of combined immunodeficiency.

We will be continuing these studies in order to clarify the many doubts that appear in this context. A deficit of this enzyme's activity could constitute a direct cause of immune dysfunction or could reflect a deviation of cellular function or a genetic change that would reach simultaneously the immune capacity and enzymatic activity.

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