原　著

THE EVALUATION OF ADA ACTIVITY IN PLEURAL EFFUSION FOR THE DIAGNOSIS OF TUBERCULOUS PLEURAL EFFUSION

Shang-Jyh KAO*, David WANG, Feng-Yee CHANG, Kang HSU
Chien-Yeh SHEN and John CHEN

(Received for publication September 21, 1987)

Pleural effusion from 59 cases of various diseases were studied by high pressure liquid chromatography (HPLC) in order to evaluate the diagnostic value of measurement of adenosine deaminase (ADA) activity. Patients were divided into 4 groups : (1) 18 cases of tuberculosis ; (2) 5 cases of bacterial empyema ; (3) 22 cases with malignancies ; (4) 14 miscellaneous group cases including uremia, cirrhosis of liver, congestive heart failure and systemic lupus erythematosus. The mean ADA activity was 110.79 ± 33.85 U/L in group 1, 207.55 ± 65.82 U/L in group 2, 33.43 ± 11.80 U/L in group 3, and 25.53 ± 9.30 U/L in group 4. Comparing the levels obtained in each group, significant differences ($p < 0.001$) were found between the tuberculosis group and both the malignancies and miscellaneous groups. The empyema group also showed significant differences ($p < 0.001$) in comparison to both the malignancies and miscellaneous groups. The sensitivity (100%) and specifity (87.8%) of the test in tuberculosis were very high when a value of more than 60 U/L was employed as a diagnostic standard.

Key words : Adenosine deaminase, High pressure liquid chromatography.

Tuberculosis still remains a major health problem in Taiwan. Almost one third of the patients with extrapulmonary tuberculosis have pleural involvement (1). The diagnosis conventionally depends upon cell count, biochemistry, culture of pleural fluid and pleural biopsy (2), (3). The data from cell count and biochemistry of pleural fluid are only suggestive of a deifferential diagnosis of pleural effusion. However, the positive rate is only 10–20% in fluid culture for tuberculous bacilli and 60–80% in pleural biopsy (4–6). A definitive diagnosis can not be made in about 20–30% of patients with pleural effusion, even after repeated pleural biopsy and cytologic examination of the pleural fluid. Delayed diagnosis or misdiagnosis in cases of pleural effusion always lead to a rapid deterioration of the patient's condition and waste time and money.

Adenosine deaminase, ADA or adenosine aminohydrolase is an enzyme of purine catabolism which catalizes the pathway from adenosine to inosine (Adenosine + $H_2O \longrightarrow$ Inosine + NH_3) (7) (Fig. 1). Its distribution is widespread in human organs (8). The level of ADA is ten

* From the Pulmonary Division, Medical Department, Tri-Service General Hospital, National Defense Medical Center, Taipei.

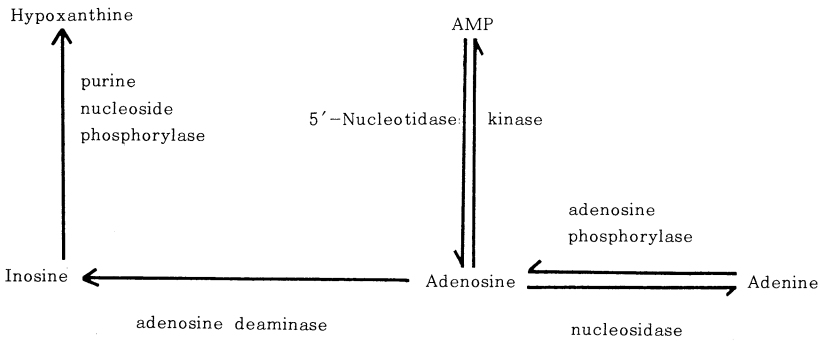

Fig. 1. Several major metabolic pathways of adenosine in biological systems.

times higher in lymphocytes than in erythrocytes, particularly during T-lymphocyte differentiation (9), (10). ADA deficiency has been reported in patients with combined immunodeficiency and an increased ADA activity was seen in typhoid fever, infectious mononucleosis, brucellosis and Mediterranean spotted fever in which cellular immunity was stimulated (9), (11-14).

In 1973, Piras reported that cerebrospinal fluid ADA Activity was higher in tuberculous meningitis than various acute or chronic inflammatory, degenerative, vascular and neoplastic diseases of nervous system (15). He provided an effective method to differentiate tuberculous from viral lymphocytic meningitis. Five years later, he also demonstrated that ADA activity was elevated in tuberculous pleural effusion (16). Further observation of ADA activity showed that it is higher in tuberculous pleural effusion than in malignant, parapneumonic and transudative effusions as reported by Ocana et al. in 1983 (17). They suggested that ADA activity is a very good parameter for diagnosis of tuberculous effusion and that it possesses high sensitivity and specifity.

There are several methods available for quantitative assay of adenosine deaminase, such as the spectrophotometric method, radioisotopic assay and colorimetric method (11-13). High pressure liquid chromatography (HPLC) has great potential for enzyme assay because several components of interest in biochemical system can be measured simultaneously (18).

In 1978, Hartwick et al presented a sensitive, optimized assay for adenosine deaminase (E. C. 3. 5. 4. 4) based on a reverse phase HPLC analysis of adenosine (19). In this method, a sample of erythrocytes was incubated with adenosine and the decrease in adenosine with time was analyzed by HPLC. We adopted reverse phase HPLC for detecting the ADA activity in pleural fluid, because it can offer several advantages over other assay methods, including improved capability to discern completing side reactions from other enzymes and can easily become employed routinely in the clinical laboratory.

Patients and Methods

Patients Selection :

Fifty-nine patients admitted to our hospital for diagnosis of pleural effusion were studied. According to the final diagnosis achieved, the patients were subdivided into four groups.

Group 1 :

Eighteen patients had tuberculous effusion diagnosed by bacteriologic and/or histologic procedures.

Group 2 :

This group included 5 patients with bacterial empyema. (three caused by staphylococcus aureus and two pseudomonas aeruginosa)

Group 3 :

This group consisted of 22 patients with malignant effusions. In all cases, diagnoses were

obtained by cytologic or histologic means. The initial tumors were localized in the lung (16 patients), breast (3 patients), stomach (2 patients) and rectum (1 patients).

Group 4 :

Fourteen patients had miscellaneous etiology (four uremic pleurisy, four cirrhosis of liver, three congestive heart failure and three lupus erythematosus).

Method of ADA Activity Determination :

Crystalline adenosine, hypoxanthine, inosine, adenine, 5′-monophosphate (Sigma), potassium dihydrogen phosphate (Merck) and LC grade methanol (Alps) were used.

The chromatograph assembly (Waters associates) consisted of one pump (model 6000), an injector (model U6K) and a UV detector (model 440). Elution was carried out on a 10 μm Nucleosil 10 C_{18} with mobile phase consisting of 14% methanol and 86% 0.01 M KH_2PO_4. A flow rate of 2.5 ml/min was used which produced a pressure drop of approximately 2000 psi. Detection wavelength throughout the study was at 254 nm.

Two hundred microliters of pleural effusion which had been centrifuged at 1500 × g for 15 minutes, was pipetted into the test tubes. Then 1000 μl of a stock buffered 3.5 mM solution of adenosine was added. The stock solution was made by dissolving a weighed amount of adenosine in a buffer which contained 0.065 M KH_2PO_4, the pH of which had been adjusted to exactly 6.83 by the addition of HCl or KOH. Following incubation for 15 minutes at 37°C, the enzymatic reactions were terminated by plunging the test tubes into a boiling water bath for 45–60 seconds. The samples were then centrifuged for 20 minutes at 20000 × g and the supernatant transferred to a clean container. Then we analyzed 5 μl of supernatant of incubation mixture and 5 μl of adenosine standard solution.

The adenosine deaminase activity was calculated by the following equation :

$$\frac{\text{STD}_{\text{conc}} \times (\text{STD}_{\text{area}} \times \frac{1000}{2000} - \text{Lysate area}) \times 1200}{\text{STD}_{\text{area}} \times 15 \text{ minute} \times 200}$$

STD conc : the adenosine concentration in adenosine standard solution.

STD area : the peak area produced by the injection of 5 μl adenosine standard solution.

Lysate area : the peak area produced by the injection of 5 μl supernatant of incubation mixture.

RESULTS

Figure 2 is the chromatogram of the 5 μl supernatant of an incubation mixture of pleural effusion and adenosine stock solution. Figure 3 showed significantly higher ADA activity in the tuberculosis (110.79±33.85 U/L) and empyema groups (207.55±65.82 U/L). Significant differences ($p<0.001$) were found between tuberculosis and malignancy (33.43±11.80 U/L) or miscellaneous groups (25.83±9.30 U/L). The empyema group also showed highly significant differences ($p<0.001$) when compared with the malignancy or miscellaneous group. Sensitivity (100%) and specifity (87.8%) of the test in tuberculosis case was very high when a value of more than 60 U/L was taken as a diagnostic standard.

DISCUSSION

When adenosine was added to the pleural fluid, the influence of several other enzymes was seen

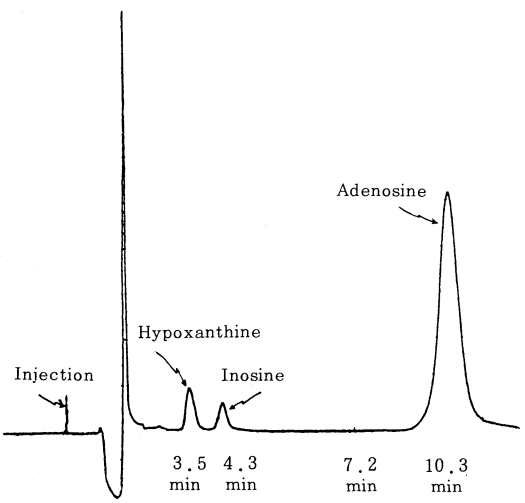

Fig. 2. Chromatogram of 5 μl supernatant of an incubation mixture of pleural effusion and adenosine stock solution.

as shown in Figure 1. Adenosine could change into inosine by ADA and thereafter change into hypoxanthine through the effect of purine nucleoside phosphorylase. There was also the possibility that adenosine changed into AMP (Adenosine Monophoshate) or adenine by 5′-nucleotidase or adenosine phosphorylase. From the data shown in Figure 2, the retention time for hypoxanthine, inosine and adenosine was 3.5 min, 4.3 min and 10.3 min. The retention time for adenine and AMP was 7.2 min and 1.3 min. Due to the fact that there were no adenine or AMP peaks in Figure 2, the peak area of 5 μl adenine stock solution is nearly equal to the sum of the peak areas of adenosine, inosine and hypoxanthine obtained from the 5 μl supernatant, we could therefore prove that there have not been other substances interferring with the assay of ADA. Therefore, we found HPLC to be a quick and specific assay method to assay the activity of ADA in pleural effusion.

Our findings seemed to confirm that ADA activity is a more effective parameter for the diagnosis of tuberculous pleural effusion. The ADA activity in tuberculous pleural effusions is much higher than that in the malignancy and miscellaneous groups. However the activity in the empyema group is particularly high ($p < 0.001$) when compared with the tuberculosis group, similar results have been reported by Yeh et al [20]. It is necessary to further determine whether it is caused by a pathogen or as the result of injured lung tissue or destroyed leukocytes.

The reason why the activity of this enzyme is high in tuberculous effusion is not clear, but it is felt that host defenses in some diseases are a function of cell-mediated immunity. ADA is a predominant T-lymphocyte enzyme and its plasma activity is high in diseases in which cellular immunity is stimulated [17], [21], [23], [24], such as in tuberculosis, typhoid fever, infectious mononucleosis [7], [8], [12]. In mononucleosis, after the entry of Epstein-Barr virus into B-cells, a strong prolonged T-cell response occurs [16]. Ocana et al. have found a high percentage of T-lymphocytes in tuberculous effusion, but this was not statistically correlated

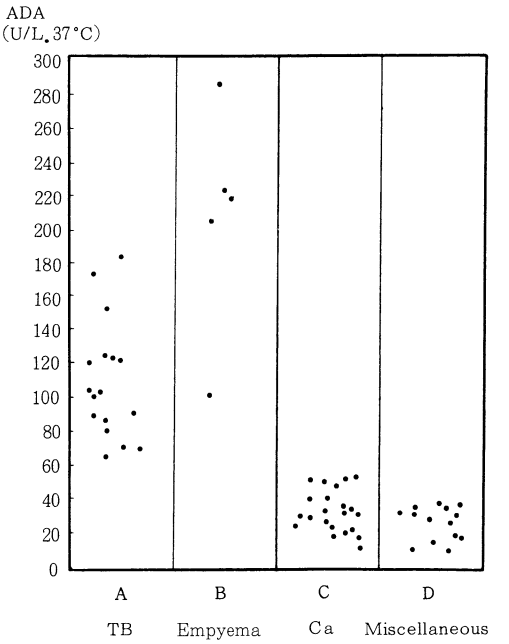

Fig. 3. Levels of ADA activity in different groups of pleural effusion

with the level of ADA [17]. This suggests that the activity of the enzyme may be correlated more to the mature stage of the T-cells than to the total number. Barton demonstrated that ADA activity varies markedly during T lymphocyte differentiation and suggested that fundamental differences in nucleotide metabolism may exist in T-cells at different stages of development [10].

Markedly higher concentrations of ADA-activity in pleural fluid than in serum suggests a local production of ADA in the pleural effusion. This may be related to a local inflammatory process within the pleural effusion and pleural membrane in which the activation of T-lymphocytes and the maturation of monocytes to macrophages is accompanied by an increase in ADA-activity [21-23].

Petterson concluded that assesment of peripheral blood and pleural fluid lymphocyte activation by phytohemagglutinin (PHA) and by purified protein derivative (PPD) yields no additional information to that obtained by conventional and laboratory procedures used in the differen-

tial diagnosis of pleural effusion [24]. Further studies are needed on T-lympocyte subpopulations, on the additional specific features of lymphocyte activation, and on the role of macrophages in the effusion.

The tuberculous and empyema groups both have high ADA-activity but which can be easily differentiated by clinical history, peripheral white blood cell count, color of pleural fluid, bacterial culture and cell count differentiation.

Increased ADA activity in rheumatoid pleurisy was also demonstrated in Petterson's observation. A reliable distinction can be made by measuring the glucose and complement (C3 & C4) concentration in the effusion [22].

In conclusion, with the aid of reverse phase HPLC, determination of ADA can be done easily and at low cost and can be performed routinely in the diagnosis of all patients with effusions, particularly if tuberculosis is suspected and in places where prevalence of this disease is still high, as is the present situation in Taiwan.

References

1) Chiang, C. H., Luch, C. S, Hsieh, Y. C. : A retrospective study of 1443 TB cases, J of Formosan Med Asso, 1981; 80 : 129-135.
2) Kao, S. J., Shen, C. Y., Hsieh, Y. C. : The evaluation of diagnosis of tuberculous pleural effusion, Chin Med J, 1983; 31 : 64-71.
3) Hirsh, A., Ruffie, P., Nebut, M., Bignon, J., Chretein, J. : Pleural effusion-laboratory tests in 300 cases, Thorax 1979; 34 : 106-112.
4) Storey, D. D., Dines, D. E., Coles, D. T. : Pleural effusion-A diagnostic dilemma, JAMA 1976; 236 : 2183-2186.
5) Berger, H. W., Mejia, E. : Tuberculous pleurisy, Chest, 1973; 63 : 88-92.
6) Chang, S. H., Perng, R. P., Pang, L. C. : Comparison of the diagnostic values of biopsys of the diagnostic values of biopsys of pleura and cytologic examination of pleural fluids, Chin Med J. 1984; 34 : 458-464.
7) Martin, D. W. Jr, Gelfand, E. W. : Biochemistry of diseases of immunodevelopment, Ann Rev Biochem, 1981; 50 : 845-877.
8) Van Der Weyden, M. B., Kelley, W. N. : Human adenosine deaminase : distribution and properties, J Biol Chem 1976; 251 : 5448-5456.
9) Gajdos, A. : Familial immune deficit and genetic deficiency of adenosine deaminase or of nucleotide phosphorylase, Nouv Presse Med 1978; 7 : 926-934.
10) Barton, R., Martinink, F., Hirschhorn, R., Goldschneider, I. : The distribution of adenosine deaminase among lymphocyte populations in the rate, J Immunol, 1979; 122 : 216-220.
11) Giusti, G. : Adenosine deaminase. In Bergmeyer, H. U., ed. Methods of enzymatic analysis, New York : Academic Press Inc, 1974.
12) Galanti, B., Nardiello, S., Russo, M., Fiorentino, F. : Increased lymphocyte adenosine deaminase in typhoid fever, Scand J Infect Dis, 1981; 13 (1) : 47-50.
13) Kalckar, H. M. : Differential spectrophotometry of purine compounds by means of specific enzymes, J Biol Chem, 1947; 167 : 461-475.
14) Piras, M. A., Gakis, C., Brudoni, M., Andreoni, G. : Immunological studies in Mediterranean spotted fever, Lancet, 1982; (8283) : 1249.
15) Piras, M. A., Gakis, C. : Cerebrospinal fluid adenosine deaminase activity in tuberculous meningitis, Enzyme, 1973; 14 : 311-317.
16) Piras, M. A., Gakis, C., Budroni, M., Andreoni, G. : Adenosine deamiase activity in pleural effusion; An aid to differential diagnosis, Brit Med J, 1978; 23-30, 1751-1752.
17) Ocana, I., Martinez-Vazquez, J. M., Segura, M., Fernaudez-DeSevilla, T., Capdevila, J. A. : Adenosine deaminase in pleural fluids. Test for diagnosis of tuberculous pleural effusions, Chest, 1983; 84 : 51-53.
18) Pennington, S. N. : 3′, 5′-cyclic adenosine monophosphate phosphodiesterase assay using high speed liquid chromatograph. Anal Chem, 1971; 43 (12) : 1701-1703.
19) Hartwick, R., Jeffries, A., Krstulovic, A., Brown, P. R. : An optimized assay for adenosine deaminase using reverse phase high pressure liquid chromatography, J of Chromatographic Science, 1978; 16 : 427-435.

20) Yeh, P. H., Lay, J. Y., Yeh, P. F., Shum Andrew, Y. C., Chen, C. F. : HPLC determination of adenosine deaminase in pleural effusions, IRCS Med Sci, 1985; 13 : 523–524.
21) Petterson, T., Klockars, M., Hellstrom, P. E., Riska, H., Wangel, A. : T and B lymphocytes in pleural effusions, Chest, 1978; 73 : 49–51.
22) Petterson, T., Klockars, M., Weber, T. : Pleural fluid adenosine deaminase in reumatoid arthritis and systemic lupus erythrematosus, Chest, 1984; 86 (2) : 273.
23) Hovi, T., Smyth, J. F., Allison, A. C., et al. : Role of adenosine deaminase in lymphocyte proliferation Clin Exp Immunol, 1976; 23 : 395–403.
24) Petterson, T., Klockars, M., Riska, H. : PHA and PPD reactivity of lymphocytes in pleural effusion, Chest, 1981; 80 : 44–47.