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DIAGNOSTIC ROLE OF ADENOSINE DEMINASE ACTIVITY IN LYMPHOCYTE RICH PLEURAL EFFUSION: A VALUABLE TUBERCULOSIS BIOMARKER

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Abstract

Tuberculosis (TB) is a chronic bacterial infection caused by mycobacterium tuberculosis which remains in dormant state for years and reactivates only when the immune system weakens or fails. Robert Koch discovered mycobacterium tuberculosis in 1882.Tuberculosis that occurs after initial exposure to the bacteria is often referred to as primary TB and is common among children up to 4 years of age and among immune-compromised persons. If TB bacteria break through the scar tissue, it results in recurrence of the pneumonia and cause active disease, referred to as secondary TB which can spread to other parts of the body like kidneys, bone, joints, peritoneum, pericardium and lining of the brain and spinal cord (meninges).This study is a step in this direction in which we tried to find out the diagnostic role of ADA in lymphocyte rich pleural effusion. This study was conducted in the department of biochemistry, Subharti Medical College, Meerut (Uttar Pradesh) .108 patients were included in the study', based on specialised diagnostic criteria patients were divided into two groups tubercular and non tubercular. The non-tubercular group was further divided into following sub groups: CHF, CRF, pneumonia, empyema, and malignancy, Light's criteria was fulfilled in all the cases to confirm the pleural fluid as exudate. All the data were recorded & statistically analysed. Out of 108 patients confirmed pulmonary tuberculosis cases were 48 in Tubercular group. Out of these patients The ADA levels were range from 5.1-27 U/L.in non tuberculous group (n=60), ADA level were ranges from 46.8-48 U/l and in tuberculous group 43.3-45.2 U/L respectively. Positive Predictive Value (ppv) and the Negative Predictive Value (npv) were calculated and were 86.6% (at 95% CI: 83.41-89.11), 85.7% (at 95% CI: 82.13-88.97) respectively.Finally it is concluded from the present study that ADA estimation in the pleural fluid is a powerful tool in the differentiation of etiology of pleural effusion and so ADA estimation has a definite diagnostic role in lymphocyte rich pleural effusion & may be used as a routine investigation for the diagnosis of such patients.

Keywords: adenosine deaminase (ADA),tuberculosis,plueral effusion,lymphocytes

Introduction

Tuberculosis (TB) is a chronic bacterial infection caused by mycobacterium tuberculosis which remains in dormant state for years and reactivates only when the immune system weakens or fails. TB kills one person every 90 sec in India and about 1000 people every day. According to WHO India account for over 20% of TB cases worldwide.[1]

The development of tubercular lesion depends upon the immune response of the body to the tubercular infection, which in turn depends on the nutritional status of the individual, concurrent use of immunosuppressive drugs and immune depleting infections like the HIV.[2]

Tuberculosis that occurs after initial exposure to the bacteria is often referred to as primary TB and is common among children up to 4 years of age and among immunocompromised persons. If the body is able to form scar tissue (fibrosis) around the TB bacteria, then the infection is contained in an inactive state. If TB bacteria break through the scar tissue, it results in recurrence of the pneumonia and cause active disease, referred to as secondary TB which can spread to other parts of the body like kidneys, bone, joints, peritoneum, pericardium and lining of the brain and spinal cord (meninges).

A pleural effusion is present when there is an excess quantity of fluid in the pleural space and develops when there is excess pleural fluid formation (from the interstitial spaces of the lung, the parietal pleura, or the peritoneal cavity) or when there is decreased fluid removal by the lymphatics.[3] The pleural fluid of tuberculous pleuritis (TP) is usually predominantly lymphocytic. Some studies suggest that pleural fluid lymphocyte percentage of more than 85% are very suggestive of tuberculosis[4]

Adenosine deaminase (ADA) is an enzyme in the purine nucleoside salvage pathway that converts adenosine to inosine. This enzyme is widely distributed in various tissues and plays an important role in the metabolism of purine nucleotides.[5] ADA deficiency leads to accumulation of adenosine and dATP; this would inhibit further production of precursors for DNA synthesis especially dCTP. The disease is caused by a mutation in a gene on chromosome 20, the gene codes for the enzyme adenosine deaminase (ADA).

The levels of adenosine deaminase (ADA) in pleural fluid offers high performance in its discriminating capacity to identify TP (sensitivity 87 to 100%, specificity 81 to 97%).[6] Only the high performance of ADA in the identification of TP allows it to be assumed that pleural biopsy can be obviated, especially in patients aged less than 35 years of age or having a lymphocyte-to-neutrophil proportion of more than 0.75 in regions of high prevalence. Quick determination and low cost justify its routine use in exudates.

Diagnosing tuberculosis early is very important to control the disease progression and prevent spread. Delay in diagnosis and in initiating treatment results in poor prognosis and sequlae in up to 25% of cases.[7]

Though the diagnosis of TB using acid fast staining of sputum smear or standard culture is considered as' Gold standard" however the sensitivity of the detection has been shown to be only 40-70%,[8,9,10], but visualization of AFB in direct smears by Zeihl-Neelsen staining requires bacillary densities of 10,000/ml and therefore detects AFB only in open cases of TB.[11]

Materials and Methods

The present study was started after obtaining the ethical clearance from the institutional ethical committee vides their letter Ref.no. SMC/EC/2010/87 dated: 27/11/2010 (Annexure 1). A,"Data collection format" (DCF) was designed (Annexure 3). Patients attending out and in-patient Departments of Tuberculosis and Respiratory Diseases of C.S.S Hospital were enrolled in the present study. Informed consent was taken in each case (copy of specimen format enclosed Annexure no. 2). The study was conducted in the department of Biochemistry, Subharti Medical College, Meerut.

General information of the patients including name, husband/father's name, age, sex, marital status, occupation, address, contact number were asked and recorded in each case.

Presenting complaints were note on DCF. Detailed present, past and family history was taken and recorded. Specific history in relation to respiratory diseases like fever and its evening rise pattern, loss of appetite, shortness of breath, dull ache, cough and expectoration were noted.

In general examination of the patients; temperature, pulse, b.p., height/weight, respiration rate, icterus, clubbing, oedema, cyanosis and any obvious lymphadenopathy were recorded. Systemic examination of central nervous system, cardio vascular system, abdomen and respiratory system were performed and positive findings noted.

During respiratory examination the signs like diminished movements, dullness on percussion, and absent breath sounds were specifically noted.

Routine investigation like Hb, TLC/DLC, ESR, GBP, CBC, Chest X-ray PA & Lateral views were done in all the cases on the basis of radiological finding.Patients were confirmed to be having pleural effusion. Ultrasound, ECG and some specialized test were performed in different patients to confirm the diagnosis.

Following diagnostic criteria was adopted for the diagnosis of the patients:

- Patients with enlarged cardiac shadow in plain X-ray chest with clinical or echocardiographic (ECG) evidence of cardiac dysfunction, with one or more of the mentioned alterations: pulmonary venous congestion on radiography, peripheral edema, tachycardia, or ventricular gallop were diagnosed as Congestive heart failure (CHF).

- Patients having raised urea and creatinine levels in the presence of clinical evidence of fluid overload (e.g., pulmonary or peripheral edema) and an absence of malignancy or respiratory infections were diagnosed as chronic renal failure.

- The presence of clinically and radiologically confirmed pneumonia with no direct or indirect evidence of bacterial presence suggested as Parapneumonic effusion.

- Patients with pneumonia along with one or more of the following indicators of bacterial invasion of the effusion: presence of pus, bacteria in Gram's stain smear or culture, and pH under 7.0 or progressively decreasing to less than 7.20 was suggested as Empyema.

- The presence of relevant auto-antibodies and clinical signs were aided in the diagnosis of Collagen vascular disease.

- Pancreatitis was suspected as chronic alcoholics, with history of severe deep boring abdominal pain and dys-electrolytemia following a bout of heavy alcohol intake, with relevant findings on abdominal ultrasound and raised serum amylase levels.

- Patients with history of smoking, persistent cough, x-ray findings, cytological diagnosis of the pleural or broncho alveolar lavage (BAL) and the elevated serum levels of specific tumor marker antigens were diagnosed as malignant pleural effusion.

- Presence of the first or any two of the other criteria must be present to label a case as tubercular;

1. Bacterial confirmation of the presence of mycobacterium tuberculosis (Direct smear or culture or histological findings in tissue sample or fluids),

2. FNAC or tissue biopsy with histopathological findings suggested of tuberculosis,

radiological findings suggestive of tuberculosis, iv) definitive clinical improvement within 2 months of anti-tubercular therapy and v) a history of contact with open cases of tuberculosis and a positive reaction >20 mm induration to 5 tuberculin unit (5TU) purified protein antigen was the adopted criteria.

Adenosine deaminase (ADA) assay: Kit used from Microxpress subdivision of TULIP Diagnostic (P) LTD.India

Reagents used in this study: MICROXPRESS ADA-MTB is a reagent for laboratory use only.ADA-MTB comprised of :

1. **L1**- ADA-MTB Reagent
2. **L2**- ADA-MTB Reagent
3. **L3**- ADA-MTB Reagent
4. **L4**- ADA-MTB Reagent
5. **S**- ADA-MTB Reagent

Principle

Adenosine deaminase hydrolyses adenosine to ammonia and inosine. The ammonia formed further reacts with a phenol and hypochlorite in an alkaline medium to form a blue indophenol complex with sodium nitroprusside acting as a catalyst. Intensity of the blue colored indophenol complex formed is directly proportional to the amount of ADA present in the sample.

$$\text{Adenosine} + H_2O \xrightarrow{\text{ADA}} \text{Ammonia} + \text{Inosine}$$

$$\text{Ammonia} + \text{Phenol} + \text{Hypochlorite} \xrightarrow[\text{Medium}]{\text{Alkaline}} \text{Blue Indophenol Complex}$$

Preparation

ADA in pleural fluid was assayed by the method of Giusti [16] with slight modifications. The technique is a colorimetric method based on the measurement of ammonia by Berthelot reaction, which is produced when ADA reacts with excess adenosine. The formation of blue indophenol was measured at 628 nm with a spectrophotometer. All solutions for the assay were prepared in house, using triple distilled water and were standardized before subjecting them for ADA estimation. Ammonium sulphate (75 µM) was used as ammonium standard. One IU of total ADA was defined as the amount of enzyme required to release 1 µmol ammonia per minute from adenosine under standard assay conditions. The enzyme is stable for at least 24 hours at 250C, for 7 days at 40C, and for 3 months at -200C [16, 17]. All the samples were stored in a deep freezer at -200C till analyzed. A positive control sample and two negative control samples for which the ADA value was known were included in each group of pleural fluid sample analyzed. The optimal cut off value of ADA was determined using the receiver operating characteristic (ROC) curve as >40 IU/L

Statistical analysis

All data are expressed as mean and standard deviations. ADA levels were measured in patients with pleural effusions due to TB and also in patients with pleural effusions of non-tuberculous origin as the controls. To compare the differences in ADA levels between the two groups, we performed the Z-test (Double-sample mean) to test the significance at 1% level of significance. Further, Pearson chi-square test without a Yates correction was also applied to find the association between the tubercular and non-tubercular groups at 1% level of significance. Also, Kruskall-Wallis test was used to compare tuberculosis versus nontuberculous groups. A two- tailed p-value less than 0.01 was considered statistically significant. On the basis of the ADA results obtained from pleural TB and control groups, the sensitivity, specificity, accuracy, predictive values, likelihood ratio and diagnostic odds ratio of the test were calculated in order to establish the potential utility of ADA as the diagnostic marker for patients with pleural TB.

Results

Table 1: Screening test result

ADA levels (IU/L)	Tubercular (n=48)	Non-tubercular (n=60)
1Positive (>40 IU/L)	39 True positive	6 False positive
Negative (≤40 IU/L)	9 False negative	54 True negative

Out of 108 patients confirmed pulmonary tuberculosis cases were 48 in Tubercular group. Out of these patients only 39 had ADA level >40 U/L (**i.e. true positive**) while 9 cases had ADA level ≤40 U/l (**false negative**). These 9 cases were previously on ATT and again got the infection (relapse cases of TB).It was the cause of low level of ADA. The ADA levels were range from 5.1-27 U/L.

In non tuberculous group (n=60), viz. CHF, CRF, Pneumonia, Empyma, and Malignant group 54 cases had ADA levels <40 U/l (**i.e. true negative**) and 6 cases had ADA level >40 U/l (**false positive**). In these 6 cases; 3 were of empyma and 3 were of malignant and ADA level were ranges from 46.8-48 U/l and 43.3-45.2 U/L respectively

Table 2: Groupwise Age, Sex and Lymphocyte % Mean ± SD

S. No.	Group	Disease	No. of patients (n=108)	Age (year) Mean ± SD	Male: Female ratio	Lymphocyte % Mean ± SD
1	**Tubercular**	Pulmonary Tuberculosis	48	35.43±16.5	13:3	88.68± 8.86
2	**Non-tubercular**	Includes diseases as below	60	50.44±19.18	19:11	85.55± 11.03
		CHF	3	25±1	3:0	92.33± 4.62
		CRF	9	58.56±17.7	2:1	70.66± 13
		Pneumonia	15	50.13±20.9	7:8	90.94± 4.52
		Empyma	9	31.4±22	2:1	86.88± 9.80
		Malignant	24	58.17±8.76	4:1	86.42± 9.56

Discussion

Pleural effusion result from disruption of this balance i.e. a pleural effusion may develop when there is excess pleural fluid formation (from the interstitial spaces of the lung, the parietal pleura, or the peritoneal cavity) or when there is decreased fluid removal by the lymphatics.[3]

Several researchers have reported that estimation of pleural fluid ADA is useful in establishing the etiology of the effusion. Shibagaki T et al concluded that tuberculous pleural effusion had a much higher ADA activity than cancer effusion.[12]

Sharma et al found that levels of pleural fluid ADA were significantly higher than serum ADA levels in both tuberculous and non-tuberculous pleural effusions.[13] Porcel J M et al found that a high ADA level is characteristic not only of lymphocytic, but also of neutrophilic TB effusions. An extremely high ADA activity should raise suspicion of empyema.[14] Gupta et al concluded that the pleural fluid ADA levels were significantly higher in tuberculous exudative pleural effusions when compared with non-tuberculous exudative pleural effusions.[15]

Lymphocyte rich pleural effusion shows neutrophil predominance in early stages and mononuclear cells later during the course of the disease. It is believed to be due to proliferation and differentiation of lymphocytes which release lymphocytes, which in turn activate macrophages for an enhanced bactericidal activity.[16]

Lee et al studied 106 nontuberculous pleural effusion samples of different etiologies, all with lymphocytic count >50% and observed that false positive test result rate is <3%; they concluded that the value of estimating ADA level not only helps in making the diagnosis in such patients, suspected of tuberculosis and also predicted that diagnosis cannot be based on total or differential leukocyte counts.[17] We also selected a cut-off of >50% lymphocytosis in pleural effusion for the present study.

L Valdes et al observed in his study of 254 patients of tuberculous pleural effusion that the mean± SD age was 34.1 ± 18.1years, and 62.2% patients were younger than 35 years.[18] these findings are similar to what we found in our study too. Epstein et al Seibert et al and Moudgil et al have reported that mean age of patients of tuberculous pleurisy gradually rises with the severity of disease.[19,20,21] While P Riantawan reported the mean age as 43 ±1.5 years but his study included HIV infected patients[22] and Wipa Reechaipichitkul reported the mean age to be higher i.e., 52.2 ±16.3 years.[23]

In the present study of 108 patients overall male: female ratio is 2.5:1; in tubercular group this ratio is 4.3:1 while in non-tubercular it is 1.7:1. (Table-2)

Sudipta et al in his study of 72 patients found male: female ratio as 1.79: 1.[23] While Wipa Reehaipichitkul in a study of 132 patients with symptomatic exudative lymphocytic pleural effusion reported the male to female ratio as 1.4:1.[24] Gupta et al found this ratio as 3:1 in his study of 96 patients with tuberculous and non-tuberculous exudative pleural effusion.[15]

Protein levels are low in our study both in pleural fluid and serum as compared to the reference normal levels. Seibert FB et al observed that moderately advanced TB of questionable clinical significance shows a decrease in albumin.[25] Damburam A. et al concluded that patients with PTB had lower serum total proteins and serum albumin but higher plasma gammaglobulin levels than controls.[26]

We calculated Positive predictive value (ppv) and the negative predictive value (npv) and they were 86.6% (at 95% CI: 83.41-89.11), 85.7% (at 95% CI: 82.13-88.97) respectively. B K Gupta et al found diagnostic sensitivity as 92.80%; 94.29%; specificity as 90.00% and 92.16%; positive predictive value 92.86% and 89.00%; and negative predictive value 90.00% and 95.92% respectively for ADA estimation though his study included both cases of pulmonary and extra-pulmonary disease.[27]

Value of ADA activity in pleural effusion was studied by Shibagaki T et al He concluded that tuberculous pleural effusion had a much higher ADA activity than cancer effusion and total ADA activity in tuberculous pleural effusion decreases after anti tubercular therapy.[28] We have also found that ADA values are less in malignant patients (24.85± 10.10) than in tubercular patients (86.17± 63.67).

The presence of a large / massive pleural effusion enables the clinician to narrow the differential diagnosis, since most effusions are secondary to malignancy or infectious (either bacterial or mycobacterial). Bloody pleural fluid with low ADA content favors a malignant condition.[29] These findings are nearly identical to those reported by Lee et al and it was concluded that measurement of the pleural fluid ADA level is an excellent test both for ruling out and ruling

in a suspected diagnosis of tuberculous effusion due to its high sensitivity and specificity, at least in areas with a high prevalence of tuberculosis.[17].

Conclusion

The present study shows that ADA estimation in the pleural fluid is a powerful tool in the differentiation of etiology of pleural effusion and so ADA estimation has a definite diagnostic role in lymphocyte rich pleural effusion & may be used as a routine investigation for the diagnosis of such patients.

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