Auxiliary tools in tuberculosis. The hemolysis in pleural fluids underestimate the values of adenosine deaminase activity determined by the method of Giusti

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ABSTRACT

The increase of adenosine deaminase (ADA) activity in pleural fluids (PF) is considered a useful tool in the diagnosis of pleural tuberculosis. It is known that numerous photometric methods are interfered by the hemolysis, as a result, hemolyzed specimens –or with blood– received in the laboratory are frequently rejected. In order to establish if the values of ADA were affected by the hemolysis or blood, ADA was determined in individual and pooled PF samples with the aggregate of erythrocyte lysate (H) or hemolyzed whole blood (HWB) from 312 mg/l to 12 g/l (final concentrations of hemoglobin in the samples), and plasma in appropriate dilutions. Negative interferences were caused by the H and HWB, starting already of 500 mg/l with relative errors until 50% in some cases, depending on the ADA activity. Increments of hemoglobin increased the negative interference. The aggregate of plasma increased slightly the ADA activity although it was insufficient for neutralize the negative effect of hemolysis. The clinical significance of the negative interference is in relation to the amount of hemoglobin present in the sample and the ADA activity. Near the cutoff (40 U/l) this interference can lead to discard erroneously the diagnosis of pleural tuberculosis.

Key words: adenosine deaminase, pleural fluids, hemolysis, interference, Giusti

INTRODUCTION

The presence of hemolysis in clinical samples is an aspect of interest in the clinical biochemistry laboratory because they can interfere in the results of numerous routine photometric methods. According with Kroll and Elin (13) interference can be defined as: "...the effect of a substance present in the sample that alters the correct value of the result, usually expressed as concentration or activity, for an analyte". This interference can significantly underestimate, overestimate or not alter the values of different parameters, depending on the degree of hemolysis, the type and amount of analyte and the method used (5, 6, 13, 18). The increase of adenosine deaminase (ADA) activity in pleural fluids (PF) is considered a very useful tool in the diagnosis of pleural tuberculosis (PTB) (1, 4, 9), usually, ADA is determined in units per liter (U/l) by the method of Giusti and Galanti, a fast, simple and economic procedure (7). This enzyme is widely distributed in the organism and it is present in appreciable amounts in lymphoid cells (19); normal values of ADA in serum have been established already of 15 U/l for normal adults and 50 U/l for children and these values can increase significantly in pulmonary tuberculosis (15, 16), especially, in
PTB (17). Frequently, PF visibly hemolyzed or with blood are received in the laboratory, and, in order to avoid possible wrong results this type of specimens are rejected. This decision is based on several considerations, it is known that the hemoglobin can interfere by absorption of light in certain wavelengths (spectral interference), also they can exists other negative interferences due to different substances from serum or by biological products released from the lysis of erythrocytes and leukocytes. On the other hand, values of ADA in the clinical sample could be falsely increased by the amounts of ADA present in serum or by the release of the enzyme as a result of the lysis of blood cells; we have found a lack of knowledge about the variation of ADA activity, when hemolysis or blood is present in the PF specimens. The aim of this work was to establish the type and degree of clinical significant interference caused by hemolysis when ADA activity in PF is determined by the method of Giusti-Galanti.

METHODS

PF specimens
Clinical samples of PF from suspected PTB patients without visible blood or hemolysis shipped to the laboratory for determination of ADA, were selected. Nine samples were classified, according the levels of ADA activity in, low (14 – 40 U/l), medium (41 – 80 U/l) and high (81 – 150 U/l) groups. Four pools were made with other samples (6 samples x pool) with values of ADA activity of 10, 25, 37, and 60 U/l, respectively. Individual PF samples and pools were aliquoted and frozened (-20 °C) until being used.

Blood
A pool of fifty milliliters of blood was obtained from 10 healthy donors and 10 ml of blood was collected from a patient with a bacteriologic confirmed PTB. In all cases, sodium citrate in standard concentrations was used as anticoagulant.

Hemolyzed whole blood (HWB)
Was obtained adding distilled water in a proportion 2/1 (water/blood) and after, by freezine (-20 °C) and thaw 3 times.

Hemolysate - as source of hemoglobin (H) - and plasma (PL)
Aliquots of blood from normal pool and from PTB patient were centrifuged (2000 g x 10 minutes), the supernatant was kept as plasma. Buffy coat was discarded and red cells were washed 3 times with isotonic saline solution; finally erythrocytes were lysated similarly that the whole blood.

Hemoglobin: concentrations and determinations
Hemoglobin concentrations greater than 1 g/l were determined and adjusted in a “Cell Dyn 1700” (Abbott Lab.) analyzer; lower values were calculated by dilutions.

Determinations of ADA
ADA activity was determined by the method of Giusti-Galanti, using reagents “Urea 2R” (Wiener Lab. Argentina); briefly, a mixture of 25 µl of sample and 500 µl of PBS adenosine solution (5.6 mg/ml) was incubated during 60 minutes at 37 °C. The reaction was interrupted by adding phenol and sodium nitroprussiate in hypochlorite solution; the mixture was incubated 10 minutes at 37 °C; the resultant indophenol released by the enzyme activity, was read at 628 nm in a digital spectrophotometer. ADA activity values were expressed in U/l (one unit of ADA correspond 3 mmol of ammonium released in 1 hour of incubation at 37 °C). Duplicate reactions were made in all cases.

Controls
PF with well-known values of ADA were used as positive and negative controls; a relative variation coefficient of 7% (20) is accepted for the method of Giusti. For controls of citrate interference, sodium citrate was added in PF samples in similar proportions that in the blood samples. ADA was determined also, in the plasma obtained from pool of normal blood and from the patient with PTB.

Hemolysis Interference studies. First set of assays
HWB, H and PL were added in different dilutions to the nine individual PF samples; HWB and H were appropriately diluted with distilled water to produce samples containing 12, 6 and 3 g/l of hemoglobin; in order to maintain the same concentrations as in the HWB. PL was added in final dilutions of 1/24, 1/12 and 1/6 respectively.

Second set of assays
Interferences were determined in four pooled PF with different ADA values (10, 39, 44 and 61 U/l) by adding diluted HWB -from the normal pool and from the TBP patient- to obtain double dilutions between 2 and 0.312 g/l of hemoglobin in the samples. Error was calculated in both relative and absolute terms, and was plotted as a function of hemoglobin concentrations. The percentage of relative error was expressed as 100 (A1-A0)/A0, where A1 is the ADA value in the presence of interferent, and A0 is the ADA value in the absence of interferent (8). The absolute error, was calculated as: A1-A0.

RESULTS

As we expected, the aggregate of sodium citrate to the samples did not produce any interference in ADA values and acceptable variation coefficients (5 - 7%) in negative (ADA 10 U/l) and positive controls (ADA 80 U/l) were obtained during the different assays. Values of ADA activity in plasma from pooled normal blood and plasma from the patient with PTB were 8 and 27 U/l, respectively. In the first set of assays, when individual samples of different levels of ADA were investigated, a negative interference caused by H and HWB was observed in all cases. This negative interference was variable according to the specimen within the corresponding group and between the different groups. When samples with similar hemoglobin values were evaluated, the negative interference caused by the aggregate of HWB was high than H. In both cases increments in the hemoglobin concentrations caused a greater interference although it was not possible to establish a proportional interference even when absolute error was calculated; in some individual samples the relative percentages of negative interference ranged 50% for H and more than 60% for HWB. A linear increment of values of absorbance was observed in the sample blanks, but not in the PF samples (figure 1). PF samples with plasma showed ADA values slightly high than controls; in some specimens this positive interference was low and no related with plasma concentrations. In the second set of experiments the negative interfe-
Hemolysis underestimate the adenosine deaminase in pleural fluids

The interference caused by endogenous or exogenous substances is a common problem in clinical laboratory. The hemoglobin is one of the four major endogenous compounds that interfere with results in the most common blood parameters (2) and there are several reports about this interferent substance, the type of analyte and the method of determination; moreover, it is known that exists analyte-dependent and analyte-independent interferences, and they can be distinguished (11-14). The first set of experiments showed that the presence of HWB or erythrocyte lysate (as a major source of hemoglobin) cause reductions in the values of the ADA activity pleural fluids. After that, different concentrations of hemoglobin (as a marker of the degree of blood presence) were tested in order to establish the minor concentration of hemoglobin that caused interference; the negative interference becomes clear and significant from 500 mg/l although in different magnitude according to the individual samples and the different levels of initial ADA values. Different mathematical corrections has been tried to solve the problem of the hemoglobin interference in various analytes (5, 14). The model of Kroll et al. (12, 14) offers a statistical treatment of interferences using a computer system, Jai and Provasek (10) proposed a simplified evaluation

**DISCUSSION**

The interference caused by endogenous or exogenous substances is a common problem in clinical laboratory. The hemoglobin is one of the four major endogenous compounds that interfere with results in the most common blood parameters (2) and there are several reports about this interferent substance, the type of analyte and the method of determination; moreover, it is known that exists analyte-dependent and analyte-independent interferences, and they can be distinguished (11-14). The first set of experiments showed that the presence of HWB or erythrocyte lysate (as a major source of hemoglobin) cause reductions in the values of the ADA activity pleural fluids. After that, different concentrations of hemoglobin (as a marker of the degree of blood presence) were tested in order to establish the minor concentration of hemoglobin that caused interference; the negative interference becomes clear and significant from 500 mg/l although in different magnitude according to the individual samples and the different levels of initial ADA values. Different mathematical corrections has been tried to solve the problem of the hemoglobin interference in various analytes (5, 14). The model of Kroll et al. (12, 14) offers a statistical treatment of interferences using a computer system, Jai and Provasek (10) proposed a simplified evaluation

**Figure 1.** Average absorbance values in sample blanks (A) and PF samples (B), when erythrocyte lysate (H) or hemolyzed whole blood (HWB) were added to specimens at different concentrations.

**Figure 2.** Negative interference (expressed as relative error) in four PF pools with the aggregate of HWB at different concentrations (pooled normal blood and blood from a patient with PTB). Initial ADA values in PF: 60, 37, 24 and 10 U/l respectively. Serum ADA in pooled normal blood: 8 U/l; in blood from PTB patient: 27 U/l.
of interferences when absolute errors greater than 10% of the initial values are clinically significant; both methods were proposed for serum analytes and for automatic analysis; but these formulas are not applicable when ADA activity in PF is determined by the method of Giusti. Interferences in the activity of adenosine deaminase appears as a complex phenomenon probably due to multiple factors; as in other analytes hemoglobin causes a interference of spectral type, since the sample blanks (without adenosine as substrate) are increased with the aggregate of hemolysate to the specimens in a proportional way, however, this increment is neutralized when the reaction takes place; the reaction is strongly inhibited by the hemoglobin or other compounds of the cellular contents. PF are biological samples with variable composition (proteins, lipids, electrolytes) and it is possible that some of them interfere directly or interact with different blood substances; also, we must have present that the method of Giusti is a manual procedure with acceptable coefficients of variation of 7%; these considerations also may explain some erratic results observed in our assays. The addition of HWB would have to increase the values of ADA in the samples as a result of the liberation of this enzyme by the lysis of leukocytes and the ADA present in serum; this increase would have to be greater when the values of ADA in serum are high. When plasma was incorporated to the samples in similar concentrations of those existing in the HWB, values of ADA were increased with little clinical relevance; we expected that ADA present in the HWB from plasma and leukocytes would neutralize the negative interference of hemoglobin, this was not observed even when we used HWB from one patient with PTB with values of 27 U/l of ADA activity in serum. Our results showed that concentrations of hemoglobin greater than 500 mg/l cause a significant inhibition of the ADA values although it was difficult to establish a linear relation. The decision for analyze hemolyzed PF specimens would have to be based on the amounts of hemoglobin present in the sample; this could be possible establishing a colorimetric scale for categorize the sample (6), or dosing the hemoglobin by a sensitive method (i.e. “Hemoglobin, Hgb, Free Plasma” Labcorp USA). The percentages of interference with clinical significance are difficult to establish; ADA in PF can be elevated in other pathologies in addition of PTB, consequently, for optimal sensitivity and specificity the negative/ positive cutoff is fixed in 40 U/l, values between 40 and 70 U/l do not allow to exclude other etiologies and values greater than 70 U/l firmly suggests PTB (3). In specimens with high or very high values of ADA the negative interference could be insufficient to change a positive result, but specimens with values near the cutoff surely will be reported as negatives leading to the clinician to a mistaken diagnosis. Moreover, the negative interference caused by the hemolysis could have more significant when ADA is determined in other type of specimens (cerebrospinal fluid or serum) with low values of ADA activity. In conclusion, the determination of ADA activity by the method of Giusti is affected by a negative interference due to hemoglobin; this interference can have a clinical relevance when the concentration of hemoglobin is 500 mg/l or more and depending on the initial values of ADA activity; the presence of plasma as contaminant is not relevant, even when levels of ADA are increased in blood. The mechanism of interference is complex and variable and probably, several substances from blood cells interacting with substances of the sample or reagents

Figure 3. Relative and absolute errors in four PF pools with the aggregate of HWB (pooled normal blood) at different concentrations. Initial ADA values in pooled specimens: 60, 37, 24 and 10 U/l respectively.)
could be involved. For practical purposes when small amounts of non hemolyzed blood are present in the sample, we recommended the centrifugation (2000 g x 10 minutes) for discard the pellet of red cells. PF specimens with visible hemolysis whose results of ADA activity are under 40 U/l would not have to be informed and the cause must be reported to the clinician. Further investigations must be realized in order to establish if the negative interference caused by the hemolysis exist when ADA is determined by other methods or when it is determined in other biological fluids.

REFERENCES