

Diagnostic Value of 5'-Nucleotidase on Kala - Azar Patients

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Summary:

J Fac Med Baghdad
2005; Vol. 47, No.3
Received July 1998
Accepted Jan. 1999

5'-Nucleotidase activity was measured in the sera of (67) Kala-azar patients before treatment and at different stages of treatment with pentostam as well as in (30) age matched normal children. The changes in 5'-NT isoenzyme profile were also followed among the above cases.

A change of the activity of the enzyme with the progress of the disease was observed. Our result suggest also that there are changes in the 5'-NT isoenzyme profile with the severity of the disease.

Introduction:

5'-Nucleotidase (5'-NT, Ec 3.1.3.5), specifically catalyzes the hydrolysis of the 5' monophosphates of purine and pyrimidine ribosides. Commonly, most tissues contain two isoenzymes of 5'-NT, the intrinsic membrane one^(2,5) and a soluble cytosolic form⁽⁶⁻¹⁰⁾.

The enzyme has important physiological functions, one of them is the production of adenosine from extracellular nucleotides^(11,13). In routine clinical chemistry practice 5'-NT has been measured in serum where its level is increased in hepatobiliary disease and malignancy⁽¹⁴⁾.

Kala-azar disease has a vast geographic distribution involving millions of people, is caused by species of leishmania (*L-donovani complex, L-chagasi, L-infantum*) which disseminate hematogenously infected macrophage in the liver, spleen, bone marrow and lymph nodes. Demonstration of the parasite, preferably by culture is essential before starting treatment^(1,5,16,17). The parasite can be demonstrated in tissue from the spleen, liver, bone marrow, lymphatic gland & less common - blood⁽¹⁾, even though this technique has a good results but complications as hemorrhage make it of less use in practice^(1,5,18,19). Many immunologic tests were performed which were based either on cellular method as in leishman - a skin test, or on humoral methods depending on the demonstration of circulating antibodies. Among these the methods mostly used in practice are immunofluorescent antibody test (IFAT) & micro-enzyme linked immunosorbent assay (ELISA) & now days, the specific monoclonal antibodies, this later method found to be positive even when the IFAT & ELISA are negativeTM.

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We have previously purified and characterized the enzyme (5' NT) from sera of untreated Kala-azar patients^{1,21}. Throughout this study we followed the changes of 5' activities and isoenzymes pattern with the severity of the disease and UDH treatment of the disease.

Material and Methods:

Thirty two untreated Kala-azar patients were included in this study, they were admitted to different pediatric hospitals in Baghdad during the period (12 / 1994 - 6 / 1995) and thirty two patients received treatment: with pentostam (20 mg / Kg) of body weight for 21 days. These patients are from different areas of Iraq and their ages ranged between 3 months - 5 years. The diagnosis was based on the clinical grounds supplemented by IFAT test and / or the demonstration of the parasite in the amastigote form in direct smear of bone marrow, or the promastigote form in culture of bone marrow. Thirty samples were obtained from healthy children, attending the maternal and child welfare units in Baghdad and were used as control for comparison. Blood samples were obtained by vein puncture from different sites depending on the age of the child involved it was collected carefully and slowly in order to avoid any hemolysis which could interfere with the results obtained.

Blood samples were left at room temperature to clot, then centrifuged at 3000 rpm for 15 minutes and the separated sera were used throughout this study.

Chemicals:

The chemicals used were from different companies and as follows: Adenosine 5'-monophosphate (5'-AMP), hydrochloric acid, stannous chloride, sodium hydroxide, sulfuric acid,

ammonium molybdate, potassium dihydrogen orthophosphate, tris-hydroxy methyl aminomethane, disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium dodecyl sulfate and magnesium sulfate were from (BDH). bovine serum albumin and hydriazine sulfate were from (SIGMA). Nickel chloric from Hopkin and Williams LTD 5'-AMP sepharose 4.B from phannacia the chemicals. Sodium barbital from (Corning ACI special).

Methods:

Separation of 5'NT isoenzyme:

Isoenzyme forms of an enzyme vary in their affinity to their substrate, so we chose affinity chromatography as a technique to separate 5' NT isoenzyme present within the sera of Kala-azar patients. And in order to do so batch sample of the sera of each group of patient used throughout this study (protein concentration of 10 mg) were applied on a 5' AMP sepharose 4 B columns (1.7x1.5 cm) pre-equilibrated with tris-HCl buffer (10 M pH 8).

Fractions of one milliliter volume were collected upon the enzyme elution from the column using 10 mM tris containing 10 mM 5' AMP (AMP buffer pH 8) at a flow rate of 0.25 ml/min.

The enzyme activity and the protein concentration were determined as described below.

Assay of 5'AT activity:

5' NT activity was measured by following Wood and Williams method (1981)⁽²²⁾. Unit of enzyme activity was defined as the amount of the enzyme that produce 1 μ mole of phosphate by hydrolysis of ester bond present in 5' AMP when potassium dihydrogen phosphate (KH_2PO_4) was used as a standard.

Protein determination:

Determination of serum protein was performed as described by Lowry et al (1951)⁽¹³⁾ using bovine serum albumin as a standard.

The specific activity of 5' NT; expressed as unit of enzyme activity / mg of proteins.

Results and Discussion:

Previous study carried out in our Laboratory⁽²¹⁾ showed that 5' NT activity in sera of normal children ranged between (2.5-11.901 U/L). An increase in the activity was observed in 41% of the Kala-azar patients studied where the activity reached up to 70 U/L...

Throughout this study we looked on the relationship between the enzyme activity and the severity of the disease in which the IFAT titre was used as an index of the disease severity. It is obvious from table (1) that 5' NT activity in the sera of Kala-azar patient changed with the disease severity.

Sample	Percentage of the Studied Cases	IFAT Titre	5'NT Activity U/L
Untreated Kala-Azar patient (32) Case	17.4%	$\frac{1}{16}$	8.9-11.9
	39.1%	$\frac{1}{32}$	11.9-30.0
	43.5%	$\frac{1}{64}$	30.0-70.0

Table (1) Relationship between 5'NT activity in the sera of patients and the severity of the Kala-azar disease.

5'-NT activity was found to be equal to the upper limit of normal (ULN) activity when the IFAT titre was (1/16) while it increased three fold the upper limit of normal (ULN) when the titre was (1/32) and a maximum 5' NT activity was observed [seven fold (ULN)] when the titre was (1/64)

These values coincide with the value obtained in cases of intrahepatic obstruction⁽⁴⁾. The increase in 5'-NT activity observed could reflect the pathological picture of non specific granuloma formation in the liver of these patients.

It has been noticed by many investigators in Iraq, that the Kala-azar patients show differences in their disease severity as well as in their response to chemotherapy^(25,26,27,28) AMP sepharose 4B has been widely used as a general ligand for affinity chromatography to separate many diverse nucleotide metabolizing enzyme which differ in their kinetics properties related to the value of K_m for AMP and its analogous compounds, and in an attempt to study the possibility of using the changes in 5' NT isoenzyme profile as a diagnostic tool to follow up the severity of the kala-azar and the progress of the treatment. Each sample of sera of the Kala-azar patient at different stages of the disease before and after their treatment, were applied on 5' AMP sepharose chromatography column and as described in the materials and methods section. The changes in isoenzyme profile were followed where it was assumed that there may be a specific elution pattern of these isoenzyme from the column depending on their K_m values for 5' AMP. In our previous study⁽¹⁷⁾, the results showed that two isoenzyme of 5' NT were present in the sera of the Kala-azar patient each present in more than one form. Throughout this work, Figures (1, 2, 3 and 4) show the changes in the 5' NT isoenzyme pattern upon elution from the column. depends on the severity of the disease. A new 5' NT peaks with low K_m for AMP appear in

the patient sera during the early stage of the disease. Upon treatment with pentostam the isoenzyme profile changes (Fig 2 A and B) toward the normal pattern (Fig. 1) and it is clear that with the treatment isoenzyme it disappears with an increase in the specific activity of isoenzyme I, when the child received the treatment at the Beginning of the infection (i.e., when IF AT titre was 1/16): while the activity of Is enzyme I increases Eighty with a decrease in one form of isoenzyme II to reach zero and a reduction of 62.5 % in the other form when the IFAT titre was (1/32) (Fig.3 A and B). When the IFAT value was (1/64). the picture seems to be different (Fig. 4 A), although four peaks of 5' NT again were obtained and the isoenzyme profile change again upon the treatment with slightly different profile (Fig. 4 B). Further work is carrying on in our laboratory to characterize these differences. The change in the 5' NT isoenzyme profile that have been reported here may reflect involvement of different organs in the infection which leads to the appearance of different isoenzyme (forms) of 5' NT in the sera of Kala-azar patients. It is known that the parasite attack the reticuloendothelial system in the liver (Kupffer cells) thus the liver paranchyme is not attacked at the beginning of the infection and this explain why some patients shows normal activity, but with the progress of the disease extensive, hypertroph and hepatomegaly, the liver paranchyme is slowly damaged so the elevation of 5' NT activity observed in 28.6 % of the cases which has high titre.

From this study we can conclude that following the changes in 5' NT isoenzyme profile may be used as 2 diagnostic tool to follow up Kala-azar infection and the progress of their Treatment. The possibility of the overall diagnostic value of 5'-NT would be changed by studying of the changes in 5'-NT isoenzyme pattern in patients suffering from different disease including hepatobiliary disease . a work being carrying out now in our laboratory .

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Legend to the figures:

Fig 1 The isoenzyme profile of 5' NT from sera of normal children. 5' NT isoenzymes were separated by applying the sera of normal children (5-10 mg protein / ml) on 5'-AMPsepharose-4B column (1.5* 1.7 cm) all details are explained in the material & method section.

Fig 2 The isoenzyme profile of 5' NT from sera of

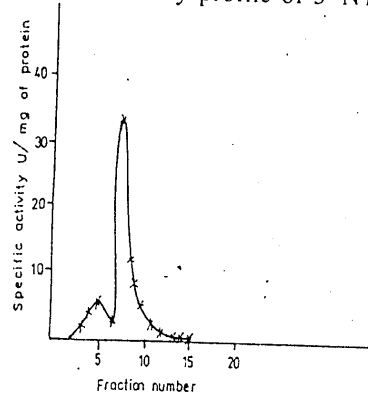


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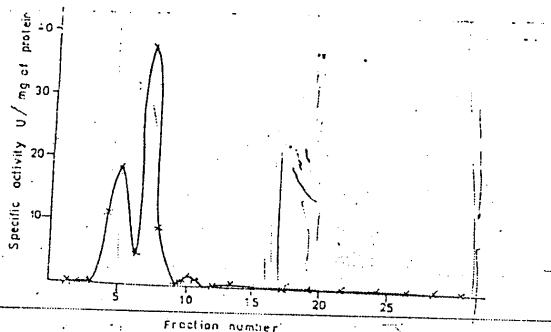
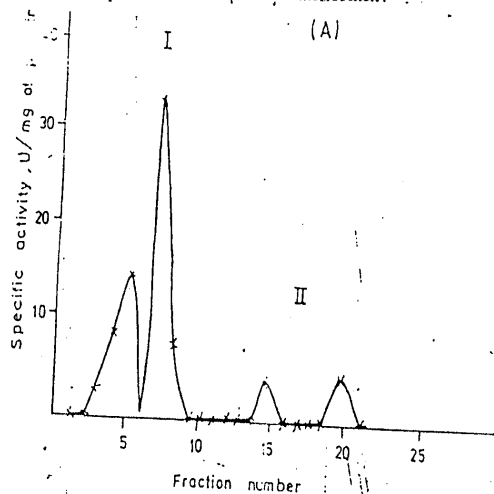


Fig.2 The isoenzyme profile of 5' NT from sera of kala-azar patient with

IFAT value of 1/16: (A) Before treatment, (B) After the treatment with pentostatin for 21 days

Lda-azar patient with IFAT value of (1/16): (A) Before treatment, (B) After the treatment with pentostatin for 21 days

Fig 3 The isoenzyme profile of 5' NT from sera of kala-azar patient with IFAT value of (1/32). (A) Untreated patient, (B) Treated patient.

Fig 4 the isoenzyme profile of 5' NT from sera of Lila-azar patient (IFAT value 1/62) (A) Untreated patient, (B) Treated patient.

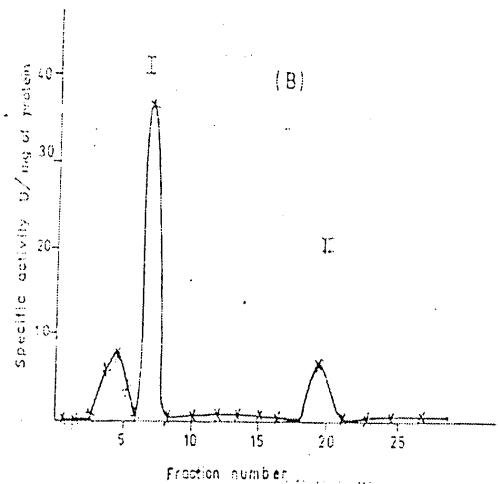
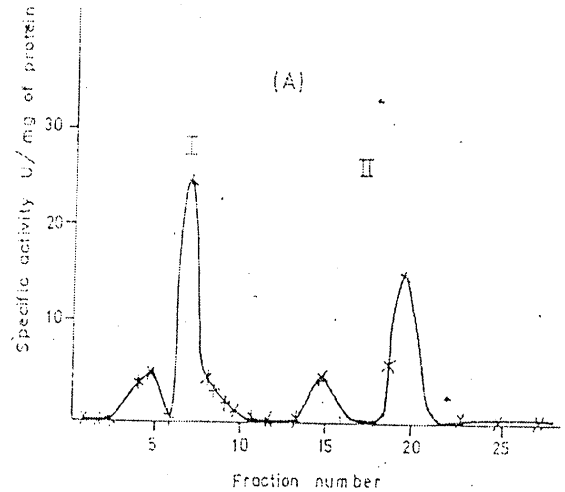


Fig.3 The isoenzyme profile of 5' NT from sera of kala-azar patient with

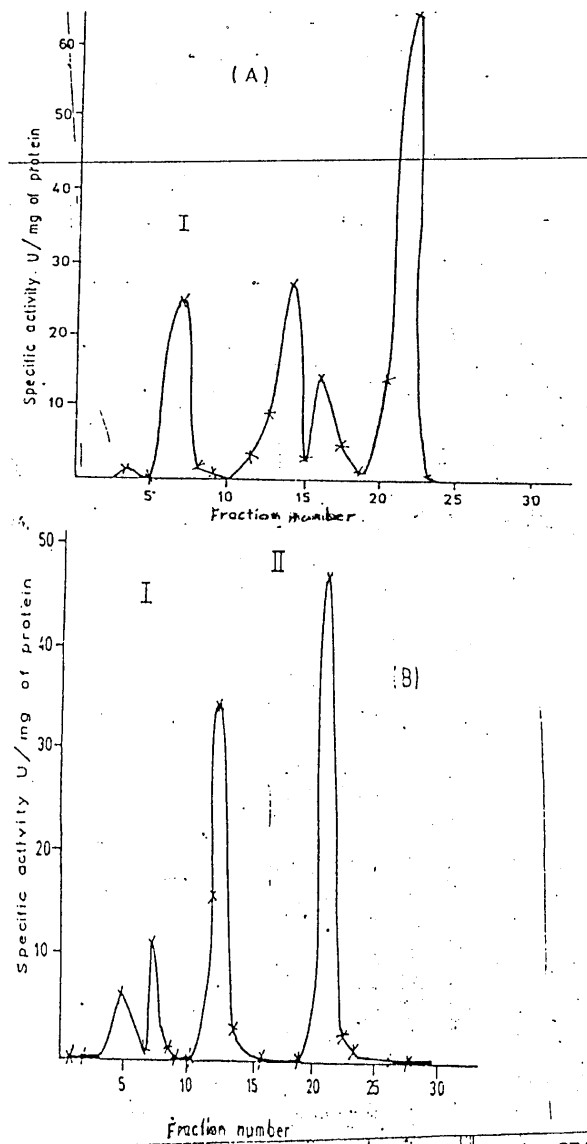


Fig 4 The isoenzyme profile of 5'NT from sera of Kala-azar patient (IFAT value) (A) Untreated patient, (B) Treated patient.