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THE ISOZYME PATTERNS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN BLOOD CELLS, BONE MARROW AND OTHER HUMAN TISSUES

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SUMMARY

The isozyme patterns of D-glucose-6-phosphate:NADP+ 1-oxidoreductase, EC 1.1.1.49 (glucose-6-phosphate dehydrogenase or Glc-6-*P* dehydrogenase) in blood and bone marrow cell lysates, and in homogenates from other human tissues were determined by the application of polyacrylamide gel disc electrophoresis.

Nucleated blood cells, including bone marrow cells, and spleen homogenates exhibited the presence of five enzymatic bands (Bands I, II, III, IV, V). Liver homogenates showed four bands (Bands II, III, IV, V), lymph nodes showed three (Bands I, II, V) and brain homogenates exhibited two (Bands I, II) bands.

In all hematopoetic cells examined, Bands I and II were found to be specific for Glc-6-*P* dehydrogenase while Bands III, IV and V seem to be due to hexose-6-phosphate dehydrogenase activity.

No differences were found in the Glc-6-*P* dehydrogenase isozyme pattern in samples of leukocytes, platelets or bone marrow from healthy subjects and from patients suffering from Glc-6-*P* dehydrogenase deficiency.

INTRODUCTION

The enzyme Glc-6-*P* dehydrogenase is of great interest because of its central position in the pentose phosphate pathway [1, 2], its involvement in various hemolytic disorders [3–6], the variation of its activity under different hormonal and nutritional states [7], and its potential as a regulator for the availability of the reduced nicotinamide adenine dinucleotide phosphate (NADP) required for various biosynthetic processes. Furthermore, this deficiency is probably the most prevalent, clinically significant, genetically determined abnormality in man [8–10]. The different forms of this disorder are inherited as a sex-linked characteristic with incomplete dominance in the heterozygous female [11, 12].

On starch gel electrophoresis, Glc-6-*P* dehydrogenase from crude hemolysates migrates as a single band but, when the source is partially purified hemolysates or leukocytes lysates from subjects who are either Glc-6-*P* dehydrogenase normal variants A+ or B+, two prominent bands appear [13]. Lymphocytes have a fast Glc-6-*P* dehydrogenase and granulocytes a slow band [14].

The enzyme appears as one band in hemolysates and leukocyte lysates when excess NADP is added to the electrophoretic run [15].

In a previous study we checked the isozyme distribution of Glc-6-*P* dehydrogenase in blood cells and in cells of different age groups taken from normal Glc-6-*P* dehydrogenase subjects, and two enzymatically active bands were found [16].

In the work herein described, we investigated the Glc-6-*P* dehydrogenase isozyme patterns in different hematopoetic cells and tissues and determined the specificity of these various isozymes with respect to phosphate substrate.

MATERIALS AND METHODS

Blood cell lysate preparation. Heparinized venous blood was taken from 120 healthy subjects and from 15 patients suffering from Glc-6-*P* dehydrogenase deficiency (Mediterranean type). The blood cells were separated and lysates of erythrocytes, platelets and total leukocytes were prepared [17]. Separated lymphocytes, polymorphonuclear granulocytes [18] and lymphocytes culture [19] lysates were prepared in a similar manner.

Bone marrow cells were obtained from 15 patients who suffered from either hematologic or malignant diseases and from 5 patients with Glc-6-*P* dehydrogenase deficiency. The bone-marrow cells in a saline heparinized solution were centrifuged at 1300 $\times$ *g* for 6 min, the supernatant fluid was removed and the sedimented cells were washed three times in cold saline. Subsequently, the sediments were resuspended in 0.5 ml saline and lysed by freezing in a mixture of acetone and dry ice and then thawing. The lysate was centrifuged at 1300 $\times$ *g* for 6 min to remove cell debris and the supernatant served as a source of the enzyme.

Tissue hemogenates. Liver, spleen, lymph nodes, muscle and brain obtained at autopsies were homogenized and lysed [17] and finally centrifuged at 20 000 $\times$ *g* for 10 min at 4 °C. The supernatant served as the source of the enzyme.

Protein content. The protein content was determined by the Biuret method [20]. The optimal volume for hemolysate electrophoresis was 15 μl containing 90 to 120 mg protein. For other blood cells, lysate and homogenates the optimal quantity for electrophoresis was 0.3 to 0.6 mg protein in a volume of 10 to 150 μl.

Electrophoresis. The electrophoretic run was carried out by disc electrophoresis on polyacrylamide gels for an hour and a half at room temperature [21]. The gel columns were removed from the glass tubes, placed in test tubes containing incubation medium and were incubated in the dark for 90 min at 37 °C [22]. The gels were rinsed with distilled water and then stored in test tubes containing 7% aqueous acetic acid.

A check of the specificity of the enzyme fractions was made by incubating the gels in the presence and absence of D-glucose 6-phosphate (Glc-6-*P*). The gels were also incubated with 6-phosphogluconic acid, D-galactose-6-phosphate (Gal-6-*P*) and 2-deoxy-D-glucose 6-phosphate (2-deoxy-Glc-6-*P*) at a concentration of 1.5 mM.

Glc-6-P dehydrogenase measurements in hemolysates and homogenates. The enzyme activity was determined spectrophotometrically [23].

6-Phosphogluconate dehydrogenase assay was done as described by Yoshida [24].

Re-electrophoresis of the enzyme fractions extracted from the gel columns. The gel was sliced after electrophoresis, the location of the unstained enzyme was determined by comparison with stained gel columns. The enzyme-containing slices were

placed in test tubes containing 50 mM Tris–maleate buffer pH 7.4 and homogenized. The homogenates were kept at 4 °C for 12–15 h and centrifuged at 20 000 × *g* for 30 min at 4 °C. The supernatants contained the extracted enzyme.

Enzyme aggregates investigation. The hemolysates were electrophoresed either in the absence of ammonium persulphate [25] or with riboflavin as a polymerising agent.

Heat lability. Heat lability was determined by incubating normal hemolysates at 56 °C for 30 min, and at 45 °C and 50 °C for 0, 5, 10, 20, 40 and 60 min. An aliquot of each of the heated samples representing 100% enzyme activity was used to determine the enzyme electrophoretic pattern.

RESULTS

Two bands, staining blue violet, were obtained on electrophoresis of erythrocyte lysate at pH 8.3. The lower anodic one, Band I, is faster and appeared below hemoglobin A_2. The upper cathodic one, Band II, is slower and stained most deeply (Fig. 1).

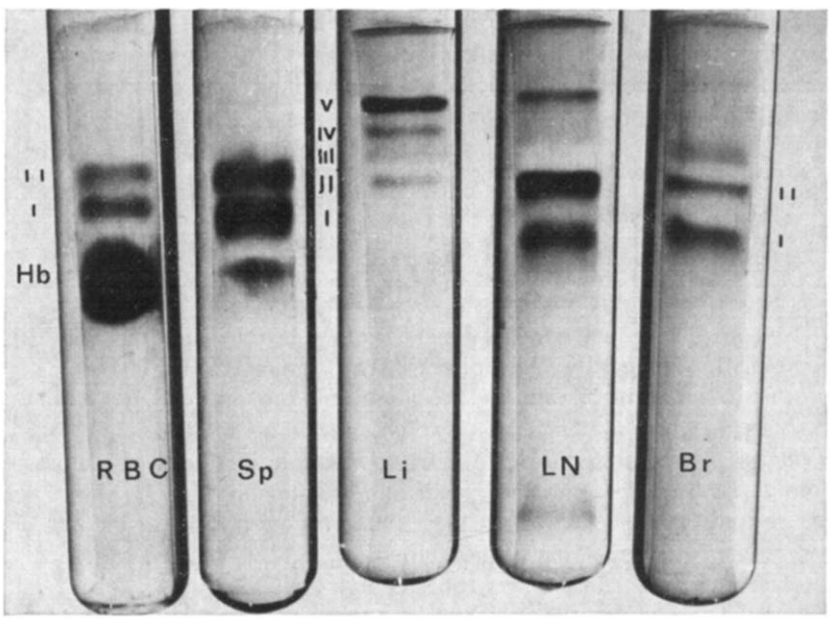

Fig. 1. Isozymes distribution of Glc-6-*P* dehydrogenase in red blood cells and tissues. A comparison between the number and location of Glc-6-*P* dehydrogenase isozymes from lysates of red blood cells (RBC) and tissue homogenates of spleen (Sp), liver (Li), lymph node (LN) and brain (Br).

Blood cell lysates

Platelet lysates from normal and from Glc-6-*P* dehydrogenase deficient subjects gave similar patterns with the Glc-6-*P* dehydrogenase activity in Bands I and II. Most samples of the leukocyte lysates from normal and deficient patients showed in addition to Bands I and II, three additional lines which stained as very thin zones.

The same pattern was obtained from separated leukocytes, venous blood lymphocytes as well as from lymphocytes which had undergone blastic transformation.

Five zones of enzymatic activity similar to those found in leukocytes were observed in bone-marrow cell lysates, Bands I and II being broad and deeply coloured, and Bands III, IV and V, narrow and less deeply coloured (Fig. 2).

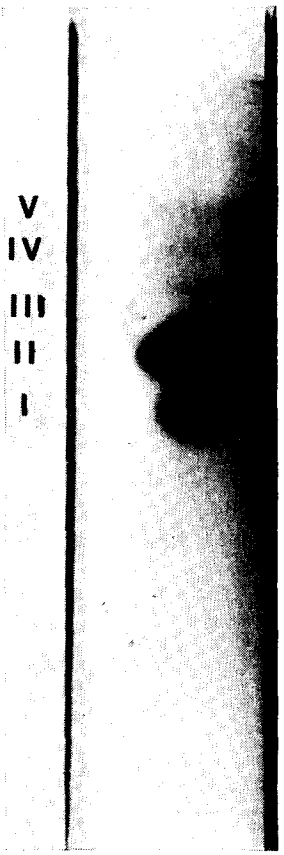

Fig. 2. Electrophoretic separation of Glc-6-*P* dehydrogenase bands from bone-marrow cells lysate. Bands I and II are wide and dark, Bands III, IV and V are narrow and light enzymatic bands.

Tissue homogenates

The isozyme pattern of Glc-6-*P* dehydrogenase in erythrocytes and in various tissues is given in Fig. 1.

Five zones of enzymatic activity similar to those found in bone-marrow cells, leukocytes and lymphocytes were observed in spleen homogenates (Fig. 1). Bands I and II were broad and deeply coloured and III, IV and V were narrow lines and less deeply coloured. Lymph-node homogenates showed Bands I, II and V. Liver homogenates showed four zones in the region corresponding to Bands II, III, IV and V for bone-marrow cell isozymes. The Bands II, III and IV were narrow and less deeply stained, and Band V was narrow and very deeply stained. Brain homogenates gave

two specific bands equivalent electrophoretically to Bands I and II of homolysate and additional fine bands were found without the addition of any substrate, perhaps because of the great endogenic content of glucose in this tissue. In muscle homogenate no positive staining was observed.

Specificity of the enzymatic bands

Using Glc-6-*P* as substrate the strong enzymatic staining of Bands I and II which appeared in hemopoetic cells and tissue homogenates except liver (Band II), indicate specifically the presence of Glc-6-*P* dehydrogenase. This is due to the fact that with the other hexose-6-phosphates as substrates a very weak staining was obtained in blood cells lysate except of mature normal red blood cells (Fig. 3).

Bands III, IV, V which were revealed in nucleated blood cells, spleen and liver appear to be nonspecific bands, since not only Glc-6-*P* but also 2-deoxy-Glc-6-*P* and Gal-6-*P* serve as substrates. Fraction V which appeared as a single non-specific one in lymph-node homogenates, is very deeply stained in this tissue and in liver homogenates (Fig. 3).

Using 6-phosphogluconic acid, no enzymatic bands were found by the method applied by us.

Re-electrophoresis of the extracted Glc-6-P dehydrogenase isozymes

When each band from the hemolysate was extracted from the gel column and re-electrophoresed, a single band was obtained at the expected position.

When the electrophoretic run was done on polyacrylamide where ammonium persulfate was omitted or where riboflavin was used, the same electrophoretic pattern was obtained.

Heat lability

The incubation of a hemolysate sample at 56 °C for 30 min caused complete Glc-6-*P* dehydrogenase inactivation. Band I was completely inhibited after 20 min incubation at 50 °C whereas Band II was markedly inhibited. At 45 °C, Band I ceased to appear after 40 min incubation whereas Band II still appeared after 60 min. When the volume applied to perform electrophoresis was increased to get the same activity as before the enzyme inactivation, two bands again appeared. This indicates that the two isozymes from normal erythrocytes showed no differences in thermal stability.

DISCUSSION

Polyacrylamide gel zones which were stained with formazan showed specific activity for Glc-6-*P* dehydrogenase in hemolysates since no staining was obtained in the absence of the substrate, Glc-6-*P*, or when 6-phosphogluconic acid, Gal-6-*P* or 2-deoxy-Glc-6-*P* were used in the incubation solution.

The two Glc-6-*P* dehydrogenase isozymes that were shown to be present in aged erythrocytes are of the same molecular weight [16]. The isozyme pattern as well, does not change on addition of β-mercaptoethanol which causes dissociation of aggregates of enzymes or NADP which prevents the dissociation of Glc-6-*P* dehydrogenase into subunits [16]. The repetition of the original isozyme pattern of Bands I and II on re-electrophoresis of samples extracted from gel columns, in addition the

fact that two bands were obtained on polyacrylamide gel prepared in the absence of ammonium persulfate which causes aggregation [25], or with riboflavin, instead of ammonium persulfate as initiator of polymerization, support the conclusion that the Glc-6-*P* dehydrogenase bands obtained in the hemolysate represent native isozymes and are not the result of association or dissociation.

Because Glc-6-*P* dehydrogenase deficiency is among the most prevalent genetic deficiencies in man, we thought it of advantage to determine its isozyme pattern in the cases where a spectrophotometric analysis showed a severe deficiency of Glc-6-*P* dehydrogenase in erythrocytes.

Since the Glc-6-*P* dehydrogenase content of erythrocytes decreases with ageing, the decrease in cases of enzyme deficiency may be more pronounced and rapid so that in aged cells no enzyme activity remains [26, 27]. On the other hand, nucleated cells (bone marrow, leukocytes, platelets and lymphocytes) do not show any difference in their isozyme pattern as compared to normal cells.

We may assume that this enzyme which has a structural abnormality [28] and does not differ in electrophoretic mobility from the normal enzyme, will show kinetic and biochemical differences [29].

Bands I and II appear to be specific for Glc-6-*P* dehydrogenase and were characterized by their strong enzymatic activity in comparison with the fine enzymatic lines found when hexose 6-phosphates other than Glc-6-*P* were used as substrates.

Bands III, IV and V are less specific since Gal-6-*P* and 2-deoxy-Glc-6-*P* serve also as substrates and the activity with respect to all three reacting substrates was found in the same position on the acrylamide gel in lysates and homogenates studied (Fig. 3). These findings are similar to the activity of hexose-6-phosphate dehydrogenase studied by Beutler and Morrison in liver [30].

Although spectrophotometrically a high activity of 6-phosphogluconate dehydrogenase was detected, no bands were demonstrable by electrophoresis; this may reflect inadequate concentration of the substrate used in the incubation solution or other methodologic factors.

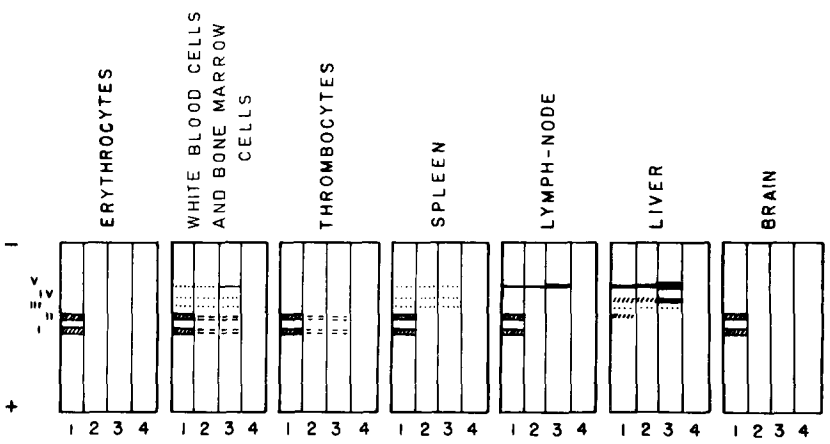

Fig. 3. Human blood cells and tissues distribution of glucose-6-phosphate and hexose-6-phosphate dehydrogenases separated on acrylamide gel electrophoresis showing the specificity of Glc-6-*P* dehydrogenase isozymes in Bands I and II. (1) Glc-6-*P*; (2) 2-deoxy-Glc-6-*P*; (3) Gal-6-*P*; (4) 6-phosphogluconic acid.

Unlike the findings of isozymes in hemopoetic tissues, no bands were found in muscle homogenates and it is correlated with the low spectrophotometrically measured activity found, indicating that the pentose-phosphate shunt does not play a major role as a source of energy in this tissue.

The different isozyme distribution of each tissue may be an expression of the adaptation of the enzyme structure to the metabolic requirements of the different cells.

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