British Journal of Haematology, 1985, 61, 41–50

Human erythrocyte hexokinase deficiency: a new variant with abnormal kinetic properties

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Received 20 September 1984; accepted for publication 9 January 1985

SUMMARY. A 14-month-old child who had a haemolytic episode when he was 5 years old, and with psychomotor retardation, was found to have decreased red cell hexokinase activity. The mutant enzyme was characterized by an increased affinity for glucose associated with an increased inhibition constant for glucose-1,6-diphosphate. Affinity for Mg ATP^{2-}, heat stability and pH-optimum were normal. The isozymic pattern of the red cell enzyme was normal but all the molecular forms were present in reduced amounts. The kinetics of decay of hexokinase during cell ageing was also normal. Glucose consumption of the hexokinase deficient cells was 60–65% of the controls while the amount metabolized through the hexose monophosphate shunt was unchanged. Red cell 2,3-diphosphoglycerate and glucose-6-phosphate levels were normal in the proband but reduced in the erythrocytes of his parents, who were heterozygous for the defect but had normal haematological data. Comparison with the 13 previously reported cases of hexokinase deficiency confirms the broad phenotypic variability that characterizes this disorder.

Red blood cells and brain are almost exclusively dependent upon glucose utilization to generate metabolic energy (Brewer, 1974; Lowry *et al*, 1964). Among all the glycolytic enzymes, hexokinase (ATP: D-hexose-6-phosphotransferase, HK) has the lowest specific activity. It is strongly modulated by many phosphorylated compounds, and is one of the regulatory steps of the glycolytic pathway (Brewer, 1974; Lowry *et al*, 1964).

The crucial role of this enzyme is well documented by the relative rarity of HK deficiency. In fact, as suggested by Paglia *et al* (1981), it might be assumed that genetically induced deficiency of hexokinase is incompatible with red cell survival. However, after the first report of true HK deficiency in 1967 (Valentine *et al*, 1967), 13 other families have been described.

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In some cases the mutant enzyme was found to show modified substrate affinities (Keitt, 1969; Moser *et al*, 1970; Necheles *et al*, 1970; Goebel *et al*, 1972; Siimes *et al*, 1979; Newman *et al*, 1980), in others reduced heat stability (Keitt, 1969; Board *et al*, 1978; Newman *et al*, 1980), or modified regulatory properties (Rijksen & Staal, 1978; Rijksen *et al*, 1983). Few papers report only a reduced enzyme activity with no additional enzyme abnormalities (Beutler *et al*, 1978; Paglia *et al*, 1981). In many cases the enzyme deficiency is associated with a direct causal relation with nonspherocytic haemolytic anaemia, but other subjects show multiple malformations and latent diabetes mellitus (Löhr *et al*, 1965; Goebel *et al*, 1972). In the present study we describe a new hexokinase deficiency with modified kinetic and regulatory properties associated with psychomotor retardation.

PATIENTS AND METHODS

Patient

B.L., aged 14 months, was born at term after a normal pregnancy with uneventful delivery. The parents were not consanguineous but both come from a town of 5000 inhabitants near Caserta. At birth the baby showed asphyxia (APGAR score = 1) and required intensive care, heart massage and endo-tracheal catheter. On the second day he had generalized convulsions and at the age of 1 month enlarged liver and spleen were observed. The baby was first hospitalized at 3 months in the Pediatric Clinic of Siena. His weight was 7·070 kg (90th centile), length 60 cm (50th centile) and head circumference 41 cm (50 centile). The baby was not interested in its surroundings, and did not follow light, did not smile or have control of the head, had increased muscular tone of the trunk and limbs with frequent opisthotonos. He had accentuated deep tendon reflexes. The liver could be felt 1 cm below the umbilicus, the spleen reached the umbilical line. Of the tests carried out, the following were normal: blood counts, creatinine, phosphorus, alkaline phosphatase, GO and GP transaminase, gamma globulins, prothrombin time, arterial pH, blood gases, lactic and pyruvic acid levels, ammonia, copper, ceruloplasmin, galactose, aminoacidogram, aminoacids and sugars by one- and two-dimensional chromatography of the urine, serology for lues, dye test for toxoplasmosis, cerebrospinal fluid, serum and CSF examination for rubella and cytomegalovirus antibodies, peripheral and medullary blood smear, examination for uronic acid, oligosaccharide or storage material in the urine. The activity of the following lysosomal enzymes in leucocytes was normal: beta-hexosaminidase A and B, alpha-galactosidase, beta-galactosidase, beta-glucuronidase, alpha-mannosidase, alpha-fucosidase, beta-glucosidase, arylsulphatase A and B, cerebroside-beta-galactosidase, sphyngomyelinase. EEG was within normal limits. Skeletal and chest X-ray were normal as well as echocardiogram and ECG. CT scan showed a modest widening of sulci and ventricles. Echography of the abdomen confirmed the presence of an enlarged liver and spleen without structural abnormalities. Ultrastructural examination of nerves and fibrocytes in the conjunctival biopsy were normal.

At 5 months the baby was admitted to another hospital where, on a basis of a low value of haematocrit (23%), a haemolytic crisis was assumed, and a blood transfusion given.

The patient was seen again at 14 months. Psychomotor retardation was still evident but

the liver and spleen had diminished in size. In order to clarify the possible previous haemolytic episode, the following tests were performed: RBC $4{\cdot}5 \times 10^{12}$/l; Hb 11·6 g/dl; haematocrit 33·9%; mean corpuscular volume 74 fl; reticulocytes 3%; total bilirubin levels 0·3 mg/dl; haptoglobins 114 mg/dl; Coombs test (direct and indirect) negative, cold haemagglutinin absent; haemolysis in hypotonic solutions normal.

Samples

All blood samples obtained from the patient and parents were collected in heparin. Control samples were obtained from healthy people and were collected in a similar manner. Glycolytic intermediates were determined by precipitating whole blood immediately after withdrawal (Beutler, 1975). All determinations were completed within 8 h from sample collection. The blood samples were filtrated through cellulose columns to remove leucocytes and platelets (Beutler *et al*, 1976). Lymphocytes were isolated from whole blood collected in heparin, by density centrifugation (30 min, 1600 rpm, 4°C) of the blood on Lymphoprep (Nyegaard & Co., Oslo, Norway). Contaminating monocytes were removed by adherence to plastic Petri dishes and the resulting cell preparation was >95% pure.

Determinations

Red and white blood cell enzymes and intermediate compounds were determined by the methods of Beutler (1975) at 37°C and 2,3-diphosphoglycerate was determined with a test kit (Boehringer Mannheim, West Germany). White cell lysates were obtained by sonification at 100 W for 20 s and 0°C in the presence of 10 mM sodium–potassium phosphate buffer, pH 7·4, containing 3 mM mercaptoethanol, 3 mM KF, 1 mM ditiothreitol, 0·4% (w/v) saponin, 0·5% (v/v) Triton X-100 and 1 mM phenylmethylsulphonylfluoride. The sonicate was centrifuged for 15 min in an Eppendorf Microfuge and the highspeed supernatant utilized for enzyme and protein assay.

Glucose metabolism. Glucose utilization by intact red blood cells and the amount metabolized through the pentose shunt were determined as previously described (Magnani *et al*, 1983).

Hexokinase isozymic pattern. 1 ml of haemolysate prepared as previously described (Magnani *et al*, 1980) was chromatographed on DE-52 columns (Whatman, Maidstone, U.K.) (0·3 × 24 cm) equilibrated in 5 mM sodium potassium phosphate buffer, pH 7·5, containing 0·25 mM glucose and 1 mM dithiothreitol. The columns were developed with 280 ml of a linear gradient of 0–0·4 M KCl in the same buffer, at 5 ml/h. Fractions of 0·7 ml were collected and assayed for HK activity. Further details of the method are reported in Stocchi *et al* (1982).

Hexokinase purification. For kinetic experiments, the red cell hexokinase was partially purified by DE-52 column chromatography. 3 ml of haemolysate were applied to a 1·2 × 4·0 cm DE-52 column equilibrated in 5 mM sodium potassium phosphate buffer, pH 7·5, containing 3 mM KF, 3 mM mercaptoethanol, 1 mM dithiothreitol, 0·25 mM glucose (buffer A). The column was washed with 3 volumes of buffer A and the enzyme eluted with 0·4 M KCl

in the same buffer. This procedure removed the bulk of haemoglobin and provided a 40–50-fold purification with complete recovery of the enzyme.

Red blood cell fractionation

Red blood cells were diluted with 1 vol of isotonic NaCl and separated into fractions of different mean age by ultracentrifugation through a density gradient of Ficoll-Triosil layers according to the method of Turner *et al* (1974) with slight modifications (Magnani *et al*, 1983).

RESULTS

Enzyme activities

Activities of red cell enzymes in normal controls, the patient and his parents are reported in Table I. The specific activity of HK of the patient was 55% lower than in the controls. All other glycolytic enzymes assayed were normal or slightly increased (pyruvate kinase and glucose-6-phosphate dehydrogenase). Since these last two enzymes are known to be cell age-dependent (Brewer, 1974), and taking into account that among glycolytic enzymes HK is the most prominently affected by the cell age (Magnani *et al*, 1979; Stocchi *et al*, 1982), we must conclude that red cells from the proband do not contain more than 30–40% of expected HK.

The parents were found to be heterozygous for the defect. They showed a red cell hexokinase activity which is about 55% of normal.

Table I. Erythrocyte enzyme activities in patient, parents and controls

	Patient	Mother	Father	Normals (10)
Hexokinase	0·44±0·05 (5)	0·53±0·04 (3)	0·53±0·04 (3)	0·98±0·16
Glucose phosphate isomerase	55·2	47·4	51·3	60·8±11·0
Phosphofructokinase	11·0	11·3	10·7	11·0±2·3
Aldolase	4·87	4·39	4·30	3·19±0·86
Triose phosphate isomerase	2500	2100	2350	2200±350
Glyceraldehyde phosphate dehydrogenase	185	171	164	200±40
Phosphoglycerate kinase	308	342	325	320±36
Monophosphoglyceromutase	11·6	18·7	15·6	18·0±3·8
Enolase	7·2	8·6	8·2	5·2±0·8
Pyruvate kinase	18·6	15·1	16·5	14·0±2·0
Lactate dehydrogenase	121	119	120	180±25
Glucose 6-phosphate dehydrogenase	11·4	10·3	10·85	8·5±2·5
6-Phosphogluconate dehydrogenase	9·7	8·0	10·8	8·2±0·5

The values are expressed as units/g haemoglobin. Values in parentheses are the number of determinations on different blood samples.

Glycolytic intermediates and glucose metabolism

The red cell glycolytic intermediates in our patient were all within normal limits. However, because of the mean younger red cell population in the proband, the levels of glucose-6-phosphate must be considered slightly lower than normal. This was confirmed by the parents' values. In fact, in the presence of normal haematologic data and decreased HK activity, the erythrocyte glucose-6-phosphate concentrations were about 30% lower than in the normal controls (Table II).

Table II. Red blood cell glycolytic intermediates*

Compound	Normal controls†	Patient	Mother	Father
Glucose-6-phosphate	28·2±3·5	26·6	18·0	19·0
Fructose-6-phospate	7·3±1·5	7·5	7·0	7·4
Fructose-1,6-diphosphate	2·4±0·5	2·8	2·4	2·9
Dihydroxyacetone phosphate	9·1±2·0	7·3	6·8	6·9
Glyceraldehyde-3-phosphate	0·4±0·2	0·4	0·5	0·5
3-Phosphoglycerate	50·0±5·1	45·2	47·3	51·2
2-Phosphoglycerate	6·5±1·5	6·0	6·2	6·9
Phosphoenol pyruvate	12·8±2·1	13·1	12·0	13·5
ATP	1180±120	1600	1300	1200
ADP	220±40	170	220	170
AMP	20±5	12	16	24
2,3-Diphosphoglycerate	4800±200	4860	3870	4270
Glucose-1,6-diphosphate	79±6	61	55	45

* All values are expressed as nmoles/ml red blood cells.
† Means ±*SD* of five values.

The red cells of the patient possessed a reduced glycolytic ability. The amount of glucose utilized was about 38–40% less than in the controls (1·5 μmol/h/ml RBC), while the amount metabolized in the hexose monophosphate shunt was normal both in the absence or presence of methylene blue (60 and 1400 nmol/h/ml RBC).

Hexokinase properties

Some properties of partially purified red blood cell hexokinase are reported in Table III. The K_m for glucose of the proband's HK was significantly reduced with respect to the controls. No differences were found in the K_m for Mg ATP, while the K_i of glucose-1,6-diphosphate was 3–4 times the normal values.

The proband's HK showed normal pH dependence of enzyme activity and normal heat stability.

Table III. Properties of partially purified hexokinase

	Patient*	Controls*
K_m glucose (mM) ($MgATP^{2-}$ = 5 mM)	0·020 ± 0·005	0·047 ± 0·004
K_m $MgATP^{2-}$ (mM) (glucose = 5 mM)	0·62 ± 0·04	0·60 ± 0·04
K_i Glu-1,6-P_2 (mM) (glucose 5 mM and $MgATP^{2-}$ 0·25–2·5 mM)	0·070 ± 0·005	0·022 ± 0·005
pH optimum (in 100 mM glycylglycine)	7·5–8·5	7·5–8·5
Heat stability at 44°C	Normal	- - -

The values are means ± SD of three determinations.
* All determinations have been performed at pH 7·20 and 30°C.

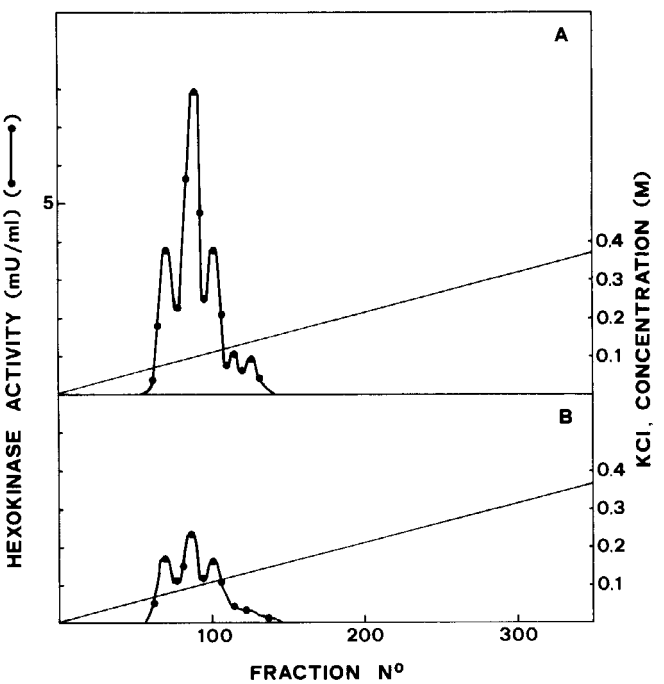

Fig 1. Hexokinase isozymic pattern in red blood cells from normal controls (A) and the proband (B). Samples of 1 ml haemolysates were applied to DE-52 cellulose columns (0·3 × 24 cm) equilibrated in 5 mM sodium potassium phosphate buffer, pH 7·5, containing 0·25 mM glucose and 1 mM dithiothreitol. The columns were developed with 280 ml of a linear gradient of KCl from 0 to 0·4 M in the same sodium potassium phosphate buffer and operated at 5 ml/h. Fractions of 0·7 ml were collected and assayed for hexokinase activity.

Isozymic pattern

Human red blood cell hexokinase has been shown to exist in multiple molecular forms (in the type I hexokinase area) which are separable by ion-exchange chromatography (Stocchi *et al*, 1982).

Chromatography of the patient's HK showed the same pattern of normal controls but all forms of the enzyme were present at a lower level (Fig 1).

Hexokinase activity and red blood cell age

It is well known that HK activity strictly depends on the red blood cell age (Magnani *et al*, 1979; Stocchi *et al*, 1982). Fig 2 shows that, in the patient, lower HK levels were found in all red blood cell fractions separated according to their age by density gradient ultracentrifugation (Turner *et al*, 1974) and that the decay rate of the proband's HK was similar to that of normal controls. The glucose-6-phosphate dehydrogenase behaviour was superimposable to the normal. The red cell distribution through the gradient (Fig 3) provided clear evidence of mild haemolytic anaemia: in fact the proportion of less dense erythrocytes (fractions 1 and 2) was markedly increased.

Hexokinase activity in lymphocytes

The total HK activity in the patient's lymphocytes was normal, as was that of the parents with values of 42, 34 and 28 mU/mg protein respectively (normal controls 40 ± 9 mU/mg protein, mean of 10 values $\pm$SD).

DISCUSSION

Red blood cell hexokinase is of central importance for erythrocyte metabolism (Brewer, 1974). This enzyme is markedly modulated by many glycolytic intermediates (Stocchi *et al*, 1982; Magnani *et al*, 1981; Ninfali *et al*, 1980) and, among all glycolytic enzymes, it shows the fastest decay during cell ageing (Seaman *et al*, 1980). In the human erythrocyte it is present in multiple forms all of which are sub-types of HK I (Fornaini *et al*, 1982). The same isozyme (HK I) is the only glucose phosphorylating activity in the mammalian brain (Wilson, 1980), while other tissues contain, in different proportions, HK type II, III and IV (Grossbard & Schimke, 1966). Red blood cell and brain are also similar because of their strict dependence on glucose utilization to generate metabolic energy, which makes HK crucial for both tissues (Lowry *et al*, 1964). By a review of the literature on hexokinase deficiency a broad phenotypic variation is evident. In some cases changes in substrate affinities have been described (Keitt, 1969; Moser *et al*, 1970; Necheles *et al*, 1970; Siimes *et al*, 1979) but not reported for other patient's enzymes (Valentine *et al*, 1967; Gilsanz *et al*, 1978; Board *et al*, 1978; Beutler *et al*, 1978; Paglia *et al*, 1981; Rijksen & Staal, 1978). Broad heterogeneity has also been described for the enzyme stability, isozymic composition, and other properties. In many reports this phenotypic variability has been considered to be a consequence of the presence of different red blood cell HK isozymes and it has been claimed that kinetic differences among them can

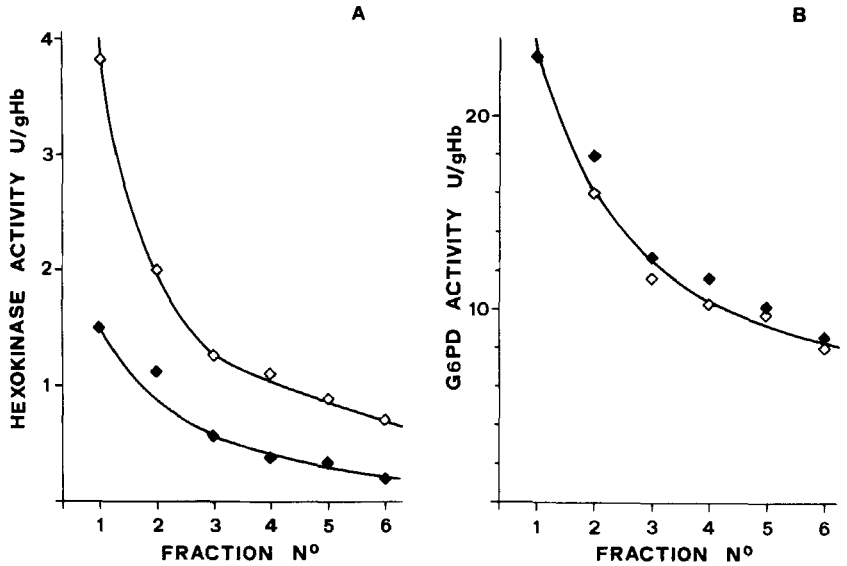

Fig 2. Hexokinase and glucose 6-phosphate dehydrogenase activities in red blood cells of different ages. Red blood cells from normal 14-month-old controls (◊) and the proband (♦) were separated into six fractions of increasing mean age by ultracentrifugation through gradients of Ficoll–Triosil. After centrifugation, red cell fractions were collected in separate tubes, washed, and haemolysed in an equal volume of 0·4% (w/w) saponin solution and assayed for hexokinase and glucose-6-phosphate dehydrogenase activities and haemoglobin concentrations. Fractions from 1 to 6 represent red cells of increasing density (age).

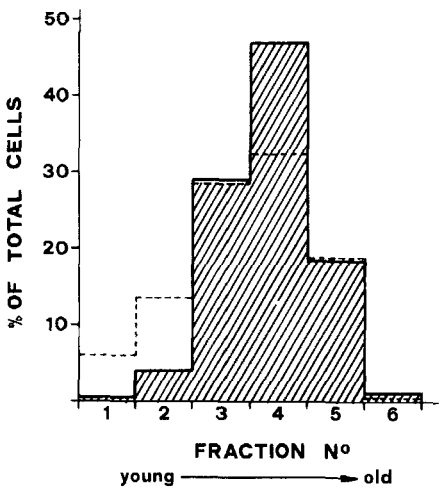

Fig 3. Separation of the proband and normal controls red blood cells into fractions of different mean ages by density gradient ultracentrifugation. After centrifugation, red cell fractions were collected into separate tubes, diluted with isotonic NaCl, and counted. Results are expressed as the percentage of cells in each fraction relative to the number of cells loaded into the gradient. Proband's red cells (– – –); controls' red cells (———).

produce phenotypic heterogeneity (Paglia *et al*, 1981; Newman *et al*, 1980; Rijksen & Staal, 1978). However, recent results from this laboratory have proved that all multiple forms of human erythrocyte hexokinase possess similar kinetic and regulatory properties (Stocchi *et al*, 1982) and that all HK isozymic forms increase in trisomy of HK gene (Magnani *et al*, 1983). The presence of post-synthetic mechanisms, which are probably responsible for HK heterogeneity (Fornaini *et al*, 1982), can help explain the differences found. In other words the normal, or mutant, gene product can be modified to different extents by post-synthetic mechanisms, resulting in different phenotypic expression of HK deficiency. Furthermore, the HK activity in the erythrocyte is markedly suppressed by different phosphorylated compounds such as glucose-6-phosphate, glucose-1,6-diphosphate, etc. In the present case, the K_i of the mutant enzyme for glucose-1,6-diphosphate is significantly increased. This fact can partially compensate for the reduced HK activity. So, it is clear that the measurement of residual HK activity is not a sufficient parameter in the study of HK deficiency. In fact, the regulatory properties of the enzyme play an important role in the determination of the glycolytic flux and this is the true parameter able to influence red cell survival.

For identification purposes, and in accordance with the recommendations of the International Standardization Committee for G6PD and pyruvate kinase deficiencies, this defect is tentatively designated HK-'Napoli'.

In the case reported the clinical picture was of severe psychomotor ratardation. One might be tempted to link the central nervous system (CNS) dysfunction to the partial HK deficiency which we have demonstrated. However, the severe neonatal asphyzia might also account for the finding. The issue of a relationship between the CNS findings and the enzymatic deficiency cannot be proved, since this might only be a coincidence. However, due to the large phenotypic variability of the HK deficiency, this possibility must be kept in mind.

ACKNOWLEDGMENT

This work was supported in part by the Cassa di Risparmio di Pesaro and Ministero della Pubblica Instruzione.

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