Clinica Chimica Acta, 140 (1984) 65–76
Elsevier

CCA 02879

Hexokinase, phosphofructokinase and pyruvate kinase isozymes in lymphocyte subpopulations

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(Received October 17th, 1983; revision March 16th, 1984)

Key words: Hexokinase; Phosphofructokinase; Pyruvate kinase; T and non-T lymphocytes; Isozymes

Summary

In order to study the three regulator enzymes of glycolysis, hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), in relation to lymphocyte maturation, lymphocytes of different origin were investigated. Lymphocytes from bone marrow, thymus, cord blood, adult peripheral blood and mitogen-stimulated lymphocytes were investigated. The enzyme activities were determined and the isozyme patterns were studied by means of electrophoresis, kinetic measurements and immunoprecipitation.

The young lymphocytes from bone marrow and the mitogen-stimulated lymphocytes could be distinguished from the other lymphocytes by a higher residual HK activity in the presence of the inhibitor glucose-1,6-diphosphate.

Peripheral blood T lymphocytes differed from non-T lymphocytes in the PK isozymes distribution. All the cells contained PK type K_4 and the hybrid K_3M. In T cells a smaller amount of the K isozyme was seen than in non-T cells. The PK residual activity in the presence of alanine was significantly higher in peripheral blood T cells than in non-T cells.

Thymocytes are characterised by a larger amount of PFK M-subunits than peripheral blood T and non-T lymphocytes. The stimulation of PFK by the positive effector glucose-1,6-diphosphate was higher in thymocytes than in the peripheral blood lymphocytes.

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Introduction

For many years lymphocytes have been the subject of intensive study by immunologists. More recently investigations have also focused on biochemical markers, e.g. enzymes [1]. Mainly enzymes of purine metabolism have been investigated [2]. In addition other enzymes, many being of lysosomal origin, have been studied [3]. Some of these enzymes are used now as markers for lymphocytes subpopulations. However, only a few reports have been published about glycolytic enzymes in the lymphocyte and its subpopulations. Lactic dehydrogenase isozymes in lymphocyte subpopulations [4,5] and phosphofructokinase [6,7] in unseparated mature lymphocytes have been investigated.

Recently, we reported on hexokinase isozyme distribution and regulatory properties in lymphoid cells [8]. It is well known that glycolysis is regulated by three regulator enzymes hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). Of these three enzymes isozymes exist, which contribute strongly to the regulatory function, because of the differences in kinetic behaviour. Four isozymes of HK are known, and designated as I to IV in order of their increasing anodal electrophoretic mobility. It is known that HK I and II can bind to the mitochondrial outer membrane. This different compartmentation is thought to provide an important regulatory mechanism by the differences in sensitivity to hexose-phosphates, some of which are strong inhibitors of HK [9]. PK consists of three isozymes, liver (L), muscle (M) and kidney (K) type. The enzyme is a tetramer; hybrid forms are known to exist. The three forms can be distinguished by electrophoresis and by kinetic measurements, such as the sensitivity to the inhibitor alanine [10]. Three isozymes of PFK are known, muscle (M), liver (L) and fibroblast (F) types. The native enzyme is a tetramer, and hybrid forms between the three isozymes can exist. The isozymes of PFK can be distinguished by kinetic measurements, such as the inhibition by ATP and the stimulation by glucose-1,6-diphosphate [11].

In this paper the isozyme distribution of HK, PFK and PK in lymphocyte subpopulations from bone marrow, thymus, cord blood, peripheral blood and mitogen-stimulated lymphocytes is reported. The isozyme patterns of HK and PK were studied with electrophoresis and kinetic measurements. PFK isozyme distribution was studied by immunoprecipitation with antibodies against the three isozymes and with kinetic measurements.

Materials and methods

Materials

ATP, ADP, $NADP^+$, fructose-6-P (all as disodium salts), phosphoenol-pyruvate (tricyclohexyl ammonium salt), glucose-1,6-P_2, fructose-1,6-P_2 (both as tetracyclohexyl ammonium salts), glucose-6-P dehydrogenase (from yeast), aldolase, glycerol-3-P dehydrogenase/triose-P-isomerase and lactate dehydrogenase were purchased from Boehringer (Mannheim, FRG). Di-isopropyl fluorophosphate was obtained from Merck (Darmstadt, FRG). Dithiothreitol, phenazine methosulphate (PMS) and bimethyl-thiazolyl-diphenol tetrazolium bromide (MTT) were from Sigma

Chemical Co. (St. Louis, MO, USA). L-α-Alanine was from BDH (Poole, UK). All other chemicals were of the highest purity available. Cellulose acetate (gelatinised) was obtained from Chemetron (Milan, Italy).

Lymphocytes

Venous blood was collected in heparin (50 U/ml), after which lymphocytes were isolated on a Ficoll-Isopaque (density 1.077 g/ml) gradient at $1000 \times g$ for 20 min [12]. Cells were used immediately or frozen as a dry pellet at −80 °C. Monocytes were removed by iron-carbonyl incubation for 1 h at 37 °C and subsequent density centrifugation. Thymocytes were supplied by Dr. H.J. Schuurman, Div. of Immunopathology, State University Hospital, Utrecht. Bone marrow lymphocytes were obtained by the method of de Gast and Platts-Mills [13] by means of a sucrose gradient. Cord blood lymphocytes were isolated essentially in the same way as the lymphocytes from peripheral blood [14]. Lymphoblasts were obtained after stimulation with phytohaemagglutinin (PHA) or pokeweed mitogen (PWM) using the method described by Gmelig-Meyling et al [15]. T–non-T cell separation was performed by spontaneous rosette formation of the T lymphocytes with sheep erythrocytes treated with aminoethyl isothiouronium bromide (AET) and subsequent density centrifugation [16].

Sample preparation

Cell lysis was performed by sonication for 30 s at 0 °C with a 150 W ultrasonic desintegrator MK 2 (MSE Scientific Instruments, Crawley, UK). The extraction buffer for hexokinase and pyruvate kinase contained 0.1 mol/l Tris-HCl (pH 7.8), 1 mmol/l $MgCl_2$, 0.1 mol/l KCl, 1 mmol/l dithiothreitol, 1 mmol/l glucose, 1 mmol/l ε-aminocaproic acid and 1 mmol/l diisopropyl fluorophosphate. The extraction buffer for phosphofructokinase where glucose-1,6-P_2 stimulation assays were carried out contained 0.02 mol/l Tris-phosphate (pH 7.5), 0.01 mmol/l fructose-1,6-P_2, 1 mmol/l $(NH_4)_2SO_4$, 10 mmol/l KF, 10 mmol/l dithiothreitol, 0.1 mmol/l EDTA and 1 mmol/l diisopropyl fluorophosphate. The extraction buffer for the PFK immunoprecipitation was 0.05 mol/l Tris-phosphate buffer (pH 7.5), containing 20 mmol/l $(NH_4)_2SO_4$, 0.2 mmol/l ATP, 0.1 mmol/l fructose-6-P, 0.1 mmol/l fructose-1,6-P_2, 1 mmol/l diisopropyl fluorophosphate, 10 mmol/l ε-aminocaproic acid, 0.1 mmol/l dithiothreitol, 10 mmol/l KF and 10 mmol/l EDTA. After cell disruption the lysate was centrifuged at $50000 \times g$ for 30 min at 4 °C. No enzyme activities could be detected in the pellet.

Enzyme assays and kinetics

Hexokinase activity was measured in the coupled glucose-6-phosphate dehydrogenase assay as described before [17]. Pyruvate kinase activity in the coupled lactate dehydrogenase reaction was measured as described by Bücher and Pfleiderer [18]. Phosphofructokinase activity was measured at pH 8.0 and 37 °C in 0.1 mol/l Tris-HCl, containing 2 mmol/l fructose-6-P, 10 mmol/l KCl, 5 mmol/l $MgCl_2$, 5 mmol/l $(NH_4)_2SO_4$, 1 mmol/l EDTA, 5 mmol/l dithiothreitol, 0.15 mmol/l NADH, 0.04% bovine serum albumin, 1.4 Katal aldolase, 1.7 Katal glycerol-3-P

dehydrogenase and 4.9 Katal triose-P isomerase. After 10 min preincubation of the sample at 37 °C the reaction was started with 0.5 mmol/l $MgATP^{2-}$. The enzyme activities were expressed in units per mg protein or units per 10^6 cells. One unit is defined as the amount of enzyme which catalyses the formation of 1 μmol product per min at 37 °C.

The inhibition of hexokinase by glucose-1,6-P_2 was determined at pH 7.15 and 37 °C as described before [8]. Inhibition of pyruvate kinase by alanine was tested at pH 7.8 and 37 °C in 0.05 mol/l Tris-HCl, containing 0.1 mol/l KCl, 1 mmol/l $MgCl_2$, 0.5 mmol/l phosphoenol-pyruvate, 0.09 mmol/l NADH, 15 Katal lactate dehydrogenase and an alanine concentration of 0.2 mmol/l. After 5 min preincubation at 37 °C the reaction was started with 0.5 mmol/l ADP. The 100% value is the activity in the absence of alanine [10]. The influence of glucose-1,6-P_2 on phosphofructokinase was studied at pH 7.4 and 37 °C in 0.05 mol/l glycylglycine containing 0.5 mmol/l $(NH_4)_2SO_4$, 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.5 mmol/l EDTA, 0.5 mmol/l $MgATP^{2-}$, 5 mmol/l dithiothreitol, 0.15 mmol/l NADH, 0.04% bovine serum albumin, 1.4 Katal aldolase, 1.7 Katal glycerol-3-P dehydrogenase, 4.9 Katal triose-P isomerase and 0.5 mmol/l glucose-1,6-P_2. The auxiliary enzymes were extensively dialysed at 4 °C before use against 50 mmol/l glycylglycine, pH 7.4, containing 0.5 mmol/l $(NH_4)_2SO_4$ and 5 mmol/l dithiothreitol. After 10 min preincubation at 37 °C the reaction was started by the addition of 0.5 mmol/l $MgATP^{2-}$. The reference value of 1.00 is the activity in the absence of glucose-1,6-P_2.

Protein determination

Protein content was measured by the method of Lowry et al, using bovine serum albumin as a standard [19].

Electrophoresis

Hexokinase electrophoresis on cellulose acetate at pH 8.8 was performed as described before [8]. Electrophoresis of pyruvate kinase was performed on cellulose acetate at pH 7.5 as was described elsewhere. Selective staining on enzyme activities was performed using MTT and PMS for colour development [20]. After visualisation of the enzyme bands the gels were scanned with a Helena Quickscan densitometer at 540 nm.

Preparation of antibodies

Preparation of antibodies against phosphofructokinase M and F types was performed by immunisation of New Zealand white rabbits by multiple subcutaneous injections of purified enzyme preparations from human muscle and human platelets [21]. The anti-muscle phosphofructokinase showed no cross-reactivity with type L and F PFK under the conditions of the immunoprecipitation assay. The purified platelet phosphofructokinase consisted of F, L and a minor fraction fo type M. The antiserum obtained against the platelet phosphofructokinase was absorbed with partially purified human liver and purified human muscle phosphofructokinase in excess. The remaining enzyme after absorption was removed from the immuno-

globulins by DEAE cellulose in 10 mmol/l K-phosphate, pH 6.8. The antibodies were subsequently precipitated with 40% $(NH_4)_2SO_4$ precipitation. A slight cross-reactivity of less than 10% with purified L and M PFK was observed. The L-type antiserum was a gift from Prof. Dr. J.F. Koster, Erasmus University, Rotterdam, The Netherlands. The anti-L serum showed no cross-reactivity with type M and F PFK under the conditions of the immunoprecipitation assay. The immunological specific activities calculated were 4 U/ml M-type antiserum, 5 U/ml F-type antibody solution and 100 U/ml L-type antiserum.

Phosphofructokinase immunoprecipitation

The immunoprecipitation was performed essentially by the method described by Cottreau et al [21]. 4–5 mU of enzyme were incubated with increasing amounts of antiserum for 1 h at 37°C, followed by incubation overnight at 4°C. The amount of rabbit serum per assay was maintained by addition of varying blank rabbit serums. After incubation polyethylene glycol was added to a final concentration of 7% and the incubation was prolonged for 15 min at 4°C. After 15 min centrifugation at 50000 × *g* and 4°C the phosphofructokinase activity in the supernatant was tested.

TABLE I

Enzyme activities of lymphocytes from different sources

Cell type	HK activity mU/mg protein	PK activity U/mg protein	PFK activity MU/mg protein
Bone marrow lymphocytes	66 ± 22 *n* = 4	2.62 ± 0.34 *n* = 4	n.d.
Cord blood lymphocytes	70 ± 38 *n* = 6	1.98 ± 0.88 *n* = 6	n.d.
Thymocytes	30, 80 *n* = 2	1.20 ± 0.36 *n* = 3	1.97 ± 55 *n* = 3
Peripheral blood lymphocytes	97 ± 48 *n* = 6	3.70 ± 0.84 *n* = 6	260 ± 64 *n* = 8
T lymphocytes	89 ± 16 *n* = 9	3.37 ± 0.81 *n* = 6	220 ± 60 *n* = 3
Non-T lymphocytes	81 ± 42 *n* = 6	2.72 ± 1.77 *n* = 5	230, 260 *n* = 2
Lymphoblasts after PHA and PWM stimulation	99 ± 30 *n* = 4	3.98 ± 1.06 *n* = 4	n.d.
Monocytes	90 ± 21 *n* = 3	3.72 ± 1.69 *n* = 4	n.d.
Granulocytes	160, 280 *n* = 2	5.71, 8.28 *n* = 2	n.d.

The values are the means ± 1 SD. The number of determinations is indicated in the Table. For details, see 'Materials and methods'.
n.d., not done.

Results

Activities

Table I summarises the activities of HK, PFK and PK from lymphocytes of several stages of maturation, monocytes and granulocytes. Hexokinase activity from lymphocytes is in the range of 40 to 140 mU per mg protein (97 ± 48 mU per mg protein, mean ± SD, $n = 6$). No differences are seen between T and non-T lymphocytes nor between lymphocytes of different sources. By comparison, granulocytes have a higher activity. Pyruvate kinase activity also did not differ for lymphocytes from the different sources or from monocytes. Cord blood lymphocytes and thymocytes show a lower PK activity, whereas granulocytes show much higher activities (5.7–8.2 U/mg protein). PFK activity from thymocytes and peripheral T and non-T lymphocytes were the same (120–400 mU/mg protein).

Electrophoresis

All the lymphocytes, as well the T as the non-T from all sources, contain only hexokinase I which is shown for lymphocytes from peripheral blood in Fig. 1. Monocytes and granulocytes show, besides hexokinase I, a HK III band (see Fig. 1). The latter may even consist of two bands. A faint hexokinase III band occasionally is seen in lymphocytes from bone marrow.

Fig. 2 shows the electrophoretic pattern of pyruvate kinase from T and non-T lymphocytes. All cell types are characterized by the presence of pyruvate kinase K_4

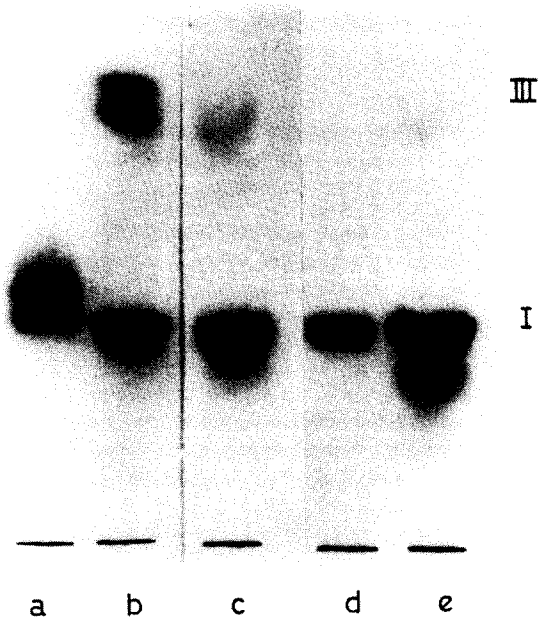

Fig. 1. HK electrophoresis from erythrocyte HK (a), granulocytes (b), bone marrow lymphocytes (c), T-lymphocytes from peripheral blood (d) and non-T lymphocytes from peripheral blood (e).

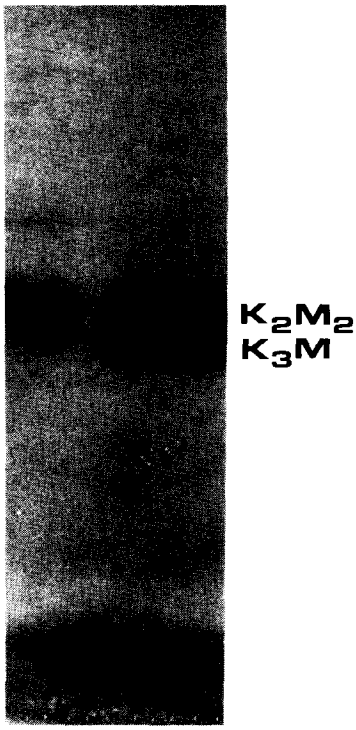

Fig. 2. PK electrophoresis from T lymphocytes from peripheral blood (left) and non-T lymphocytes from peripheral blood (right).

and K_3M. In T lymphocytes from peripheral blood a relatively small amount of the K isozyme is present, with an equal amount of the K_4 and K_3M, whereas a small K_2M_2 band could also be discerned (not visible in Fig. 2). In non-T lymphocytes the K_4 isozyme predominates, with a smaller amount of the K_3M hybrid. In granulocytes and monocytes, type K_4 is predominant besides a little K_3M (not shown).

Kinetic studies

Glucose-1,6-P_2 is a well-known inhibitor of hexokinase. Table II gives the HK residual activities in the presence of 0.1 mmol/l glucose-1,6-P_2 from lymphocytes of different sources, monocytes and granulocytes. The 100% value is the activity in the absence of glucose-1,6-P_2. The young lymphocytes from bone marrow and the lymphoblasts obtained after stimulation of lymphocytes with mitogens have a distinctly higher residual HK activity in the presence of glucose-1,6-P_2 than the lymphocytes from cord blood, peripheral blood and thymocytes. HK residual activity from monocytes was of the same order as the HK residual activity from normal lymphocytes, whereas granulocytes have a higher HK residual activity. Table II also shows the residual activity of PK in the presence of 0.2 mmol/l alanine for T and non-T lymphocytes from peripheral blood. The residual activities are expressed as percentages of the 100% value in the absence of alanine. Residual activity in T

TABLE II

Influence of effectors on enzyme activities from lymphocytes from different sources

Cell type	Residual activity of HK at 0.1 mmol/l glucose-1,6-P_2 (in %)	Residual activity of PK at 0.2 mmol/l alanine (in %)	Activation factor of PFK at 0.5 mmol/l glucose-1,6-P_2 (in %)
Bone marrow lymphocytes	59 ± 11 $n = 4$	22 ± 6 $n = 4$	n.d.
Cord blood lymphocytes	47 ± 8 $n = 6$	32 ± 14 $n = 12$	n.d.
Thymocytes	35, 44 $n = 2$	24,27 $n = 2$	3.7 $n = 1$
Peripheral blood lymphocytes	41 ± 4 $n = 33$	26 ± 6 $n = 21$	2.25 ± 0.30 $n = 28$
T lymphocytes	44 ± 5 $n = 10$	39 ± 10 $n = 13$	2.7, 3.1 $n = 2$
Non-T-lymphocytes	40 ± 6 $n = 8$	20 ± 8 $n = 10$	2.3 $n = 1$
Lymphoblasts after PHA and PWM stimulation	70 ± 11 $n = 7$	32 ± 11 $n = 7$	n.d.
Monocytes	38 ± 9 $n = 4$	20 ± 5 $n = 4$	n.d.
Granulocytes	52 ± 1 $n = 3$	21 $n = 1$	n.d.

The residual activities of HK and PK in the presence of glucose-1,6-P_2 and alanine, respectively, are expressed as percentages of the 100% value in the absence of the inhibitor.
The stimulation factor of PFK in the presence of glucose-1,6-P_2 is expressed as V/V_0, where V is the PFK activity in the presence of 0.5 mmol/l glucose-1,6-P_2 and V_0 the activity in the absence of the effector.
The values are means $\pm$ 1 SD. The number of determinations is indicated in the Table.
For details of the assays, see 'Materials and methods'.
n.d., not done.

lymphocytes is significantly higher than in non-T lymphocytes ($p < 0.005$ with Student's t test), and slightly higher than in thymocytes. All the other cell types are characterised by a relatively low PK residual activity in the presence of alanine (see Table II). The residual activity of PK is highest in cord blood (32%), and lowest in monocytes and granulocytes (20 and 21%, respectively). Finally Table II shows the stimulation of PFK by 0.5 mmol/l glucose-1,6-P_2. The activation ratios are calculated from the initial rate velocities. The V_0 value is the PFK activity in the absence of glucose-1.6-P_2. The PFK stimulation is not different in T lymphocytes and non-T lymphocytes, whereas it is slightly higher in thymocytes.

TABLE III

PFK immunoprecipitation of lymphocytes from different sources

Cell types	Residual activity of PFK (in %) in the presence of:		
	anti-M	anti-L	anti-F
Thymocytes	62 ± 4 $n = 4$	17 ± 1 $n = 3$	39 ± 8 $n = 4$
Peripheral blood lymphocytes	75 ± 7 $n = 10$	22 ± 6 $n = 5$	45 ± 12 $n = 4$
T lymphocytes	76 ± 6 $n = 3$	15 ± 4 $n = 3$	51 ± 5 $n = 3$
Non-T lymphocytes	76, 76 $n = 2$	10, 14 $n = 2$	55, 56 $n = 2$
Lymphoblasts after PHA and PWM stimulation	78 ± 9 $n = 5$	n.d.	n.d.

The 100% value is the activity in the absence of antibody. The values are means $\pm$ 1 SD. The number of determinations is indicated in the Table. For details, see 'Materials and methods'.
n.d., not done.

Immunoprecipitation

Immunoprecipitation with antibodies against M (muscle), L (liver) and F (fibroblasts) type PFK was performed on PFK from lymphocytes from peripheral blood, separated T and non-T lymphocytes, and thymocytes. The results are summarized in Table III. The results are expressed as the residual activities in the supernatant after immunoprecipitation with excess of antibody. No differences were found between the isozyme compositions of T and non-T lymphocytes. A greater amount of M- and F-containing hybrids were found in thymocytes, compared to peripheral blood T lymphocytes.

Discussion

Lymphocytes are known to originate from a bone marrow lymphoid stem cell, which differentiates into two major lymphocyte populations, designated T and B lymphocytes. T cell differentiation takes place in the thymus. After thymic maturation the T cell enters the blood-stream, where it makes up about 70% of total lymphocyte number. The T cell can proliferate after antigenic stimulation and differentiate into T effector cells, which reside in the peripheral lymphoid organs. The B cell differentiation takes place in the bone marrow. The mature B lymphocyte in the peripheral blood makes up less than 10% of total lymphocyte number. The B cell can proliferate and differentiate into a plasma cell, which resides in the bone marrow, through several stages of lymphoblasts and plasma blasts in the lymphoid organs.

Isozymes, as specific gene products, may be efficient markers of cell types,

enabling definition of a cell in terms of its molecular composition. Isozyme shifts may correlate with special metabolic functions of the differentiated tissues and may be useful in this way to relate metabolic function to morphological change. It is well known that glycolysis is regulated by the enzymes hexokinase, phosphofructokinase and pyruvate kinase. The three regulator enzymes can exist in multimolecular forms; the isozymes do not differ only in physico-chemical properties such as electrophoretic mobility, but also show different regulatory properties. Kester et al [22] and Hume et al [23] reported that energy metabolism in the lymphocyte is correlated with cell function. Glycolysis is also important for purine metabolism, which is essential for lymphocyte function [24]. Because B and T lymphocytes differentiate in a different way to highly specialised cells, isozyme changes may be involved; furthermore, during maturation of the different cell types, changes in metabolism may occur. It is therefore important to study isozyme patterns in separated T and non-T (B + 'null') lymphocytes in order to compare mature peripheral blood T cells with immature T cells in the thymus and mature peripheral blood B cells with immature B cells in the bone marrow [13].

From the results presented, it seems that with respect to hexokinase no difference exists between T and non-T lymphocytes. In both cell types only hexokinase type I could be detected. However, immature B lymphocytes show higher residual activity in the presence of glucose-1,6-P_2 than the more mature lymphocytes. Furthermore the T lymphoblasts (after PHA stimulation) have a significant higher residual activity than peripheral blood T cells. Therefore, although the electrophoretic pattern is not different, it is quite well possible that hexokinase type I varies in the different cell types. As we already reported [25], several subtypes of hexokinase type I are known, which may be differently regulated, especially with respect to the inhibition by glucose-1,6-P_2.

Pyruvate kinase can exist in three multimolecular forms, designated as K, L and M type. The K type may be regarded as the so-called fetal type; the younger the cell, the more K subunits can be detected. The latter can be easily demonstrated by means of the alanine inhibition assay, because of a direct correlation between the amount of K subunits and the alanine inhibition [20]. Thus, in young lymphocytes, both bone marrow lymphocytes and thymocytes, K_4 is predominant together with the hybrid K_3M. However, for mature lymphocytes from peripheral blood a difference between T and non-T lymphocytes could be detected. Both cell types contain type K_4 and the hybrid K_3M. However, in T lymphocytes a small amount of the hybrid K_2M_2 is present, together with a relatively smaller amount of K_4. The presence of the hybrid K_2M_2 in T lymphocytes may be important as a cell marker. Moreover, mature T lymphocytes from peripheral blood seem to contain more K_2M_2 than immature T cells from the thymus.

With respect to phosphofructokinase isozyme distribution, no differences were found between T and non-T lymphocytes from peripheral blood. By means of immunoprecipitation against the three isozymes of PFK no significant differences were found. In thymocytes, however, the amount of M- and F-containing hybrids is slightly higher. Thus, of the three regulator enzymes studied, PFK cannot be used to discriminate between T and non-T cells, and only small differences are found

between immature and mature lymphocytes of this enzyme. On the other hand, the influence of glucose-1,6-P_2 on hexokinase activity is different from the enzyme at different stages of maturation and may in this respect be a good marker for maturation of the lymphocyte. Pyruvate kinase isozyme pattern is different in mature T and non-T lymphocytes; this is not valid for the immature lymphocyte.

Acknowledgements

We are grateful to Dr. M.V.A.M. Kroeks, Dept. of Gynaecology, Diakonessenhuis, Utrecht, The Netherlands, for supplying the cord blood samples and to Ir. J. Klugkist for help with some experiments. Miss J. Kuypers and Dr. L.J.M. Spaapen are thanked for supplying some lymphocyte samples and Mrs. E.L. Huisman-Backer Dirks for typing the manuscript.

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