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HEXOKINASE ISOZYME DISTRIBUTION AND REGULATORY PROPERTIES IN LYMPHOID CELLS

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Summary

The glycolytic enzyme hexokinase is studied in cultured leukemic lymphoblasts, in normal lymphocytes and in lymphoblasts obtained by stimulation of normal lymphocytes with phytohaemagglutinin.

Hexokinase activity levels in cultured lymphoblasts and in normal lymphocytes are identical, but somewhat higher levels are found in stimulated lymphocytes. Cultured leukemic lymphoblasts differ in isozyme content in comparison to the other lymphoid cells. Besides hexokinase I, which is detected in all the lymphoid cells, they are characterized by the presence of hexokinase II. The concentration of type II increases during cell growth. Another difference between leukemic lymphoblasts and mature and stimulated lymphocytes is found in the regulatory properties of hexokinase I. Hexokinase I from both normal and stimulated lymphocytes is inhibited by glucose-1,6-diphosphate. This inhibition is decreased in part by addition of inorganic phosphate. Hexokinase I from leukemic lymphocytes, however, is inhibited to a lesser extent by glucose-1,6-diphosphate. Inorganic phosphate has no effect at all on this inhibition.

In accordance with these findings a different pattern in the hexokinase I region was detected in electrophoresis with several cell types. The subisozyme hexokinase Ib, which appears to be the phosphate-regulated form, is predominant in lymphocytes, whereas it is present in a minor fraction in the cultured leukemic lymphoblasts. In these cells hexokinase Ic predominates.

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Introduction

It is well known that changes occur in protein synthesis in tumor cells in comparison to normal cells. Alterations in membrane proteins and in plasma immunoglobulins have been observed. Also changes in hormone production and in synthesis of enzymes were reported. With respect to the synthesis of enzymes there are several possibilities. Firstly, a fully normal enzyme may be produced in larger amounts in a tumor in comparison to normal tissue. In that case only a rise in maximal activity is seen [1]. Secondly, a decrease in maximal activity of an enzyme may be found in certain types of neoplasia. Thirdly, a shift in isozyme distribution may occur. This shift is demonstrated for many enzymes, amongst which many are enzymes of glucose and purine metabolism [2]. Fourthly, in tumors an enzyme may exist which is altered in regulatory properties. Of this phenomenon only a few examples are known [3—5].

Changes in isozyme pattern and maximal activity of the enzyme hexokinase (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1) in tumors are reported [3,6—10]. Four isozymes of this important regulator enzyme of glycolysis are known: they are designated as I to IV in order of increasing anodal electrophoretic mobility [11]. Hexokinase IV is the liver-specific glucokinase.

In leukocytes, hexokinase I and III are normally present [12], whereas in lymphocytes only hexokinase I is found [13]. Stimulation of lymphocytes to lymphoblasts by phytohaemagglutinin does not result in change of the hexokinase isozyme pattern, whereas the maximal hexokinase activity increases [13].

Hexokinase, the rate-limiting enzyme of glycolysis, is strongly regulated by phosphorylated hexoses. Glucose-1,6-diphosphate, which is one of these, is a well known inhibitor of hexokinase type I [14,15]. Inorganic phosphate (P_i) is capable of overcoming this inhibition in part [15—17]. Three subisozymes of hexokinase I, designated Ia, Ib and Ic [17], may exist. It is hypothesized, that Ic is formed from Ib by a posttranslational modification. In this hypothesis, hexokinase Ib should be sensitive to P_i with respect to the effect on the inhibition of sugar phosphates, while Ic is P_i-insensitive. The three subisozymes can be separated by electrophoresis.

We studied the isozyme distribution and the regulatory properties of hexokinase in a cultured leukemic lymphoblast cell line, in normal lymphocytes and in lymphocytes stimulated by phytohaemagglutinin. The aim of this study was to provide better insight into the changes on the molecular level in cancer cells in relation to normal cells.

Materials and Methods

Chemicals

ATP (disodium salt), NAD^+, $NADP^+$ (disodium salt), glucose-1,6-diphosphate (tetra-cyclohexylammonium salt) and glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides* and from yeast) were purchased from Boehringer (Mannheim, F.R.G.).

Di-isopropylfluorophosphate was obtained from Merck (Darmstadt, F.R.G.). DEAE-cellulose (DE 52) was from Whatman (Maidstone, U.K.). Bovine serum

albumin, dithiothreitol, phenazine methosulphate and bimethyl-thiazolyl-diphenyltetrazolium bromide were from Sigma Chemical Co. (St. Louis, MO). Acrylamide and bisacrylamide were from Ega-chemie (Albuch, F.R.G.), cellulose acetate strips from Chemetron (Milan, Italy) and Gelman High-Resolution buffer from Gelman Inst. Co. (Ann Arbor, MI). Minimal essential medium and foetal calf serum were purchased from Gibco (Grand Island, NY). Hepes was from Serva (Heidelberg, F.R.G.). Agar noble special was from Difco (Detroit, MI). All other chemicals were of the highest purity available.

Hexokinase activity assay

Samples were obtained by sonication of the cells for 30 s at an amplitude of 50 μ with a 150 W ultrasonic disintegrator Mk2 (MSE, U.K.) in 0.1 M Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM di-isopropylfluorophosphate, 1 mM glucose and 1% Triton X-100. Cell debris was removed by centrifugation for 2 min at 12 800 $\times g$ (20°C). Hexokinase activity was determined at pH 7.8 at 37°C in 0.04 M Tris-HCl. Glucose concentration was 10 mM, ATP concentration 5 mM, $[Mg^{2+}]_{free}$ kept at 5 mM. The reaction was coupled to the glucose-6-phosphate dehydrogenase reaction, either with 1.5 I.U. glucose-6-phosphate dehydrogenase from yeast and 0.4 mM $NADP^+$ or with 1.5 I.U. glucose-6-phosphate dehydrogenase from *L. mesenteroides* and 0.4 mM MAD^+. The hexokinase activities were the same using both systems. 1 unit of hexokinase activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol glucose-6-phosphate per min at 37°C. Initial rate measurements were performed by following the reduction of respectively NAD^+ or $NADP^+$ spectrophotometrically at 340 nm. The K_m values for glucose and $MgATP^{2-}$ were determined at a constant Mg^{2+} concentration of 5 mM. The determinations of the K_i value for glucose-1,6-diphosphate were carried out at pH 7.15 at 37°C. Protein content was determined by the method of Lowry et al. [18], using bovine serum albumin as standard.

Cell culture

The CEM line, a continuously growing human T-lymphoblast cell line of leukemic origin, was grown in RPMI 1640-suspension, supplemented with 10% dialysed foetal calf serum. Normally, cells are passed to a fresh medium after 48—72 h with a starting concentration of $3 \cdot 10^5$ cells/ml. Cells were harvested by centrifugation at 700 $\times g$ for 10 min.

Lymphocyte isolation

Normal lymphocytes were isolated from fresh donor blood collected in ACD * buffer, as anti-coagulant, by centrifugation on Ficoll-Isopaque (density, 1.077) at 1000 $\times g$ for 20 min. The lymphocytes were carefully collected from the interface and treated with ammonium chloride for 10 min at 0°C and subsequently centrifuged at 400 $\times g$ for 10 min. Tonsillary lymphocytes were isolated by extraction of the tissue by vigorous shaking in minimal essential medium, whereafter tissue particles were removed by centrifugation at 100 $\times g$ for 30 s. This procedure was repeated three times. The pooled lymphoid cells

* ACD, acid citrate/dextrose.

were treated in the same way as the lymphocytes from blood. The tonsils were obtained from young children by routine tonsillectomy. For the preparation of monocyte-free lymphocytes, these cells were first treated with iron powder for 10 min at 37°C under gentle shaking before isolation on Ficoll. Stimulation of normal lymphocytes by phytohaemagglutinin was carried out in medium with 20% human serum of a donor with bloodgroup AB. Cells were stimulated at 37°C at a CO_2 concentration of 5% for four days. The stimulation resulted in 95% lymphoblasts.

Isozyme preparation

Samples for chromatography were extensively dialysed against 0.01 M Tris-HCl, pH 7.0, at 4°C, containing 10 mM glucose and 1 mM dithiothreitol. The sample was applied to a DE-52 column (length 20 cm, diameter 2.5 cm) equilibrated in the same buffer. Hexokinase activity was eluted with a linear KCl gradient from 0.0–0.5 M.

Electrophoresis

Electrophoresis on cellulose acetate was carried out at 4°C and 20 V/cm (±2 mA/strip) during 45 min in a Tris-Veronal buffer (Gelman High-resolution buffer) of pH 8.8 ($I \approx 0.05$), to which 1 mM glucose, 0.05 mM dithiothreitol and 1 mM EDTA was added.

Staining for hexokinase activity was carried out with essentially the same solution as used for the enzyme activity assay. Staining at a glucose concentration of 0.2 mM and 0.1 M was performed to identify hexokinase III and to detect hexokinase IV, if present. Hexokinase III has a low K_m for glucose and is inhibited at high glucose concentrations. The reaction was coupled to phenazine methosulphate and dimethylthiazolyldiphenyltetrazolium bromide-tetrazolium. 0.015 mg/ml KCN was added in order to prevent negative staining by superoxide dismutase. The staining solution was gelatinized with 1% agar. Strips were stained by laying them upside down on top of the gel in the dark at 37°C for about 30 min. Blanks stains in the absence of either glucose or ATP were carried out for control staining. Polyacrylamide gel electrophoresis using a discontinuous buffer system was performed as described previously [17]. Staining was carried out in the same mixture as used for the staining of the cellulose acetate strips with omission of the agar.

$f(P_i)$

The effect of P_i on the inhibition of glucose-1,6-diphosphate was measured as follows: $MgATP^{2-}$ concentration was 0.5 mM, the other concentrations were the same as in the maximal activity assay. Glucose-1,6-diphosphate was added to a concentration of 0.1 mM. P_i was varied from 0.2—10.0 mM. The assay was performed at pH 7.15 at 37°C. Higher pH values and variations in temperature and glucose concentration gave essentially the same effect of P_i, although inhibition of glucose-1,6-diphosphate at higher pH values was less extensive. Series of assays with increasing P_i concentration in the presence or absence of glucose-1,6-diphosphate were performed. The influence of P_i was expressed as a function of P_i calculated as described previously [19]. The function $f(P_i)$ delivers the factor by which K_i glucose-1,6-diphosphate is multiplied at a given concentration of P_i.

Results

Hexokinase activity

The average hexokinase activity for CEM cells cultured in a normal growth cycle was 0.08 ± 0.02 U/mg protein (mean ±S.D., $n = 6$). The activity was not dependent on the stage of culturing. Hexokinase activity in normal lymphocytes was 0.09 ± 0.03 U/mg protein (mean ±S.D., $n = 5$). No difference in hexokinase activity was detected between lymphocytes from peripheral blood and from tonsils.

After removal of the monocytes by the addition of iron powder the maximal activity of the monocyte-free lymphocyte preparation was the same. The hexokinase activity in stimulated lymphoblasts was 0.12 ± 0.04 U/mg protein (mean ±S.D., $n = 3$).

Disruption of the cells by 30 s sonication in the presence or absence of Triton gave essentially the same enzyme activity values. The same result was obtained with and without extraction with toluene. This result indicates that in the assay system all the hexokinase activity is solubilized (see Table I).

Isozyme distribution

The hexokinase isozyme distribution at different stages of culturing is shown in Fig. 1. In standardly harvested CEM cells, 2 days after passage into fresh medium, hexokinase I and II are present in a ratio of about 7 : 3. This ratio of hexokinase I : II appears to be dependent on the stage of growth. Growing the cells with a starting concentration of $3 \cdot 10^5$ cells/ml for 12—72 h in fresh medium results in an increasing amount of hexokinase II. On cellulose acetate electrophoresis a large amount of hexokinase activity does not migrate away from the origin. However, when the cell extracts are centrifuged at 50 000 × g for 60 min no activity is detected at the origin, indicating that a large amount is particle bound. Electrophoresis with samples which are treated with Triton is

TABLE I

ACTIVITIES AND KINETIC PARAMETERS OF HEXOKINASE IN LYMPHOID CELLS

Values are given as means ± standard deviation

	Normal lymphocytes	Phytohaemagglutinin-lymphoblasts	CEM cells	
			Type I	Type II
K_m for glucose	0.072 ± 0.016 mM ($n = 5$)	0.074, 0.089 mM ($n = 2$) *	0.084 ± 0.014 mM ($n = 8$)	0.22 ± 0.05 mM ($n = 4$)
K_m for $MgATP^{2-}$	0.56 ± 0.03 mM ($n = 5$)	0.45, 0.53 mM ($n = 2$) *	0.48 ± 0.10 mM ($n = 6$)	0.53 ± 0.07 mM ($n = 4$)
K_i for glucose-1,6-disphosphate	50 ± 28 μM ($n = 5$)	60, 85 μM ($n = 2$)	108 ± 36 μM ($n = 5$)	40 ± 15 μM ($n = 3$)
$f(P_i)$ at $P_i = 5.0$ mM	1.9 ± 0.2 ($n = 11$)	1.6 ± 0.2 ($n = 3$)	1.0 ± 0.2 ($n = 15$)	—
Maximal specific activity (units/mg protein)	0.09 ± 0.03 ($n = 5$)	0.12 ± 0.04 ($n = 3$)	0.08 ± 0.02 ($n = 6$)	

* Values are for $K_{m,app}$ at saturating substrate concentrations of the cosubstrate.

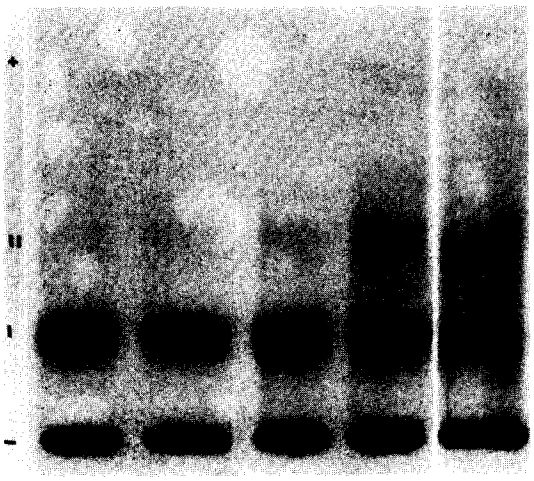

Fig. 1. The electrophoretic pattern on cellulose acetate of CEM hexokinase of cells harvested, respectively, 12, 24, 36, 48 and 60 h after passage to fresh medium (from left to right).

not possible because the sample spreads after application to the gel. Although the isozyme distribution changes during cell growth, maximal activity remains the same. The appearance of hexokinase II in human cancer cell lines, e.g. cultured hepatoma cells, has previously been shown [6].

Fig. 2 shows the hexokinase isozyme distribution for normal lymphocytes and for CEM cells, harvested two days after passage to a fresh medium. In contrast to normal lymphocytes and stimulated lymphoblasts, CEM cells contain besides type I also type II hexokinase.

The two isozymes from CEM cells were separated on DEAE cellulose. Chromatography was carried out in 0.01 M Tris-HCl, pH 7.0, with a linear gradient. Two hexokinase activity peaks were obtained. The first fraction, which eluted at 0.3 M KCl, had a K_m for glucose of 0.084 ± 0.014 mM (mean ±S.D., $n = 8$) and a K_m for $MgATP^{2-}$ of 0.48 ± 0.10 mM (mean ±S.D., $n = 6$). This is consistent with the data known for type I [19—23]. Also, the elution of hexokinase type I at 0.3 M KCl is consistent with previously reported data [23]. The second peak of activity eluted at 0.45 M KCl. It has the characteristics of hexokinase type II. The K_m for glucose was found to be 0.22 ± 0.05 mM (mean ±S.D., $n = 4$), whereas for $MgATP^{2-}$ the K_m values was 0.53 ± 0.07 (mean ±S.D., $n = 4$). These date are in agreement with other reports on type II hexokinase [21]. Hexokinase of normal lymphocytes was chromatographed under the same conditions as used for the CEM hexokinase. One peak was eluted at a KCl concentration of 0.3 M. Determination of the kinetic parameters resulted in a K_m for glucose of 0.072 ± 0.016 (mean ±S.D., $n = 5$) and a K_m for $MgATP^{2-}$ of 0.56 ± 0.03 mM (mean ±S.D., $n = 5$). These data are indicative for hexokinase I. In stimulated lymphoblasts only hexokinase I is present, as could be demonstrated by determination of kinetic data and electrophoresis. A $K_{m,app}$ value for glucose of 0.082 mM was determined at a $MgATP^{2-}$ concentration of 5.0 mM, and a $K_{m,app}$ value for $MgATP^{2-}$ of 0.49 mM at a glucose concentration of 10.0 mM was obtained (Table I).

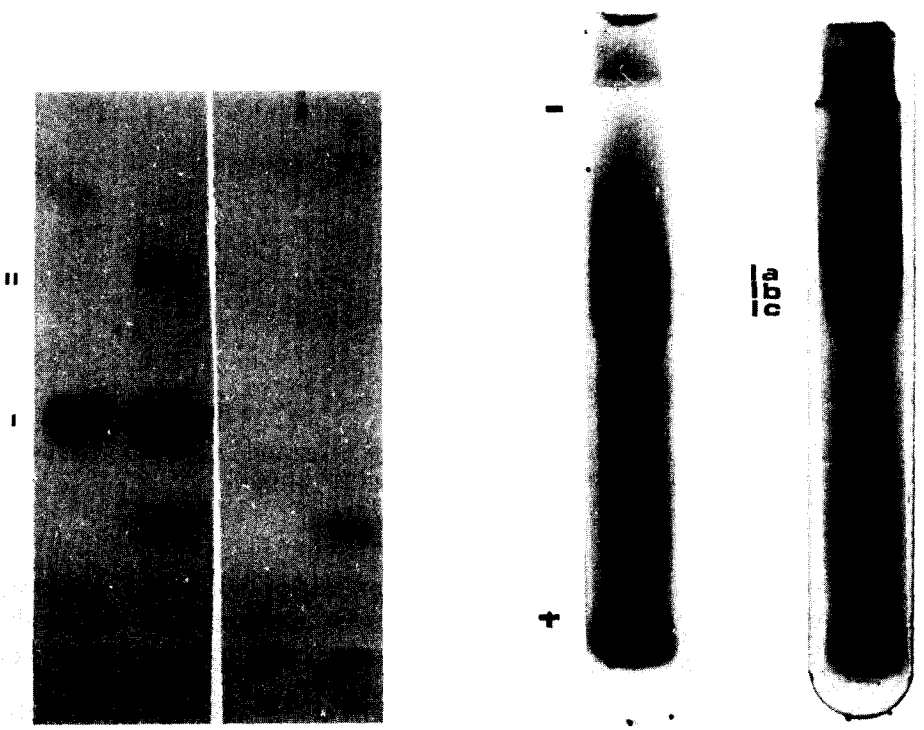

Fig. 2. Electrophoresis of hexokinase on cellulose acetate from normal lymphocytes (A) and CEM cells (B) after centrifugation of the samples at 50 000 × *g*. Blank staining without ATP is shown for normal lymphocytes (C) and CEM cells (D).

Fig. 3. Electrophoresis on polyacrylamide discs of hexokinase I (a, b and c) from lymphocytes (left) and CEM cells (right).

Several subisozymes in the hexokinase I region were detected in the different cell types using polyacrylamide gel electrophoresis. In Fig. 3 this is shown for normal lymphocytes and for CEM cells. In normal lymphocytes three subisozymes are present, whereas in CEM cells only two bands can be demonstrated. The poor separation between the bands does not allow exact identification of the particular isozymes. CEM cell hexokinase I consists mainly of two subisozymes which are presumably type Ia and Ic, according to the nomenclature of Rijksen and Staal [17]. In normal lymphocytes as well as in stimulated lymphoblasts two main bands and a minor one were present. According to the electrophoretic mobility these should be Ia and Ib for the two main bands and Ic for the minor band.

Electrophoresis on cellulose acetate resulted into at least two bands for lymphocytes. The separation was not good enough to detect a possible third band. With ion-exchange chromatography no separation in distinct fractions in the hexokinase I region was obtained.

Inhibition by glucose-1,6-diphosphate

Hexokinase is strongly regulated by inhibition by hexose phosphates such as

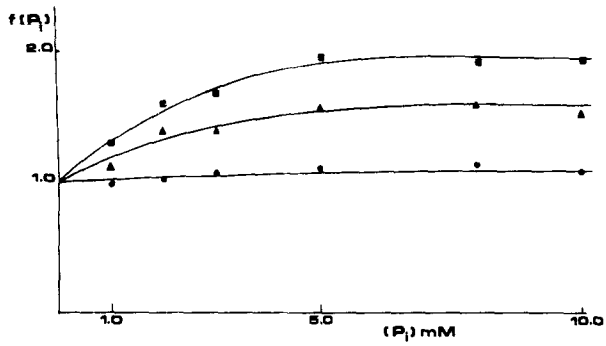

Fig. 4. Influence of P_i on the inhibition constant for glucose-1,6-diphosphate of hexokinase I from normal lymphocytes (■), phytohaemagglutinin-lymphoblasts (▲) and CEM cells (●).

glucose-6-phosphate and glucose-1,6-diphosphate [14,16,19,24]. P_i is able to abolish partly this inhibition of type I hexokinase [16,19]. Because glucose-1,6-diphosphate is thought to be an important regulator of glycolysis we determined the K_i for glucose-1,6-diphosphate and studied the effect of P_i on this inhibition. The influence of P_i on the inhibition was expressed in a formula which is described in Materials and Methods. K_i values for glucose-1,6-diphosphate at pH 7.15 at 37°C are determined for CEM hexokinase I and II and for hexokinase I of lymphocytes and stimulated lymphoblasts. For CEM hexokinase I a K_i is found of 108 ± 30 μm (mean ±S.D., n = 5). The inhibition is competitive with respect to $MgATP^{2-}$. For hexokinase II a K_i for glucose-1,6-diphosphate of 40 ± 15 μM (mean ±S.D., n = 3) is found. Lymphocyte hexokinase I is found to have a K_i for glucose-1,6-diphosphate of 50 ± 28 (mean ±S.D., n = 5), with a competitive inhibition towards $MgATP^{2-}$. For stimulated lymphoblasts the K_i for glucose-1,6-diphosphate was determined to be 73 ± 13 μM (mean ±S.D., n = 2). The kinetic data are summarized in Table I.

Influence of P_i

A difference between hexokinase of CEM cells and normal lymphocytes and stimulated lymphoblasts is found with respect to the phosphate regulation, as expressed in the $f(P_i)$ value. The $f(P_i)$ for CEM hexokinase is about 1 for all P_i concentrations, indicating that there is no influence of P_i on the K_i for glucose-1,6-diphosphate. Fig. 4 shows representative data out of 15 experiments. For lymphocyte hexokinase the $f(P_i)$ was about 2 at P_i concentrations of 5.0 mM and higher. This value is comparable with the data obtained for erythrocyte hexokinase [15]. For stimulated lymphoblasts hexokinase I a $f(P_i)$ value of about 1.6 at a P_i concentration of 5.0 mM is reached. Under the assay conditions a $f(P_i)$ value of 2.0 indicates a 2-fold increase of the K_i for glucose-1,6-diphosphate. Therefore lymphocyte and stimulated lymphoblast hexokinase I resemble erythrocyte hexokinase, whereas hexokinase from CEM cells is distinct.

Discussion

In the present paper the electrophoretic and kinetic behaviour of hexokinase in cultured leukemic lymphoblasts, normal lymphocytes and stimulated

lymphoblasts by phytohaemagglutinin is investigated. A marked difference is the presence of hexokinase II in the leukemic lymphoblasts. This isozyme is absent in the other lymphoid cells. For brain, liver and uterus tissue, hexokinase II has been reported to be a tumor marker [6—8,22], the presence of which was even described to correlate with malignancy for brain tumors [6]. Most of the other reports on hexokinase isozymes and cancer deal with hepatomas [3,10,22].

In normal lymphocytes only hexokinase I was reported to be present [13]. We were able to confirm this. In leukocytes, besides hexokinase I, hexokinase III was detected also [12]. The changing amount of hexokinase II in the CEM cells during cell culture might be an intrinsic phenomenon dependent on the cell cycle. Another possibility might be that a factor might be produced or, alternatively, might become depleted, which influences the formation of hexokinase II. Knowing that glucose can be an important factor for cell growth [25], we determined the glucose consumption in relation with the increase of hexokinase II. No correlation was detected.

The P_i-induced restoration of hexokinase activity at inhibition by phosphorylated hexoses is an important regulatory mechanism for glycolysis [15, 16,19].

Wu [26] and Kosow et al. [16] studied the influence of P_i on inhibition by glucose-6-phosphate in Ehrlich ascites tumor cells. They found no difference between the neoplastic and the normal cells. The presence of an altered regulation of gene expression, resulting in increased glycolysis by elevated enzyme levels, was stated to be a general phenomenon in cancer cells by Weinhouse [27] and Weber [28,29]. However, there are only a few reports available on changes of regulatory properties of particular enzymes of glycolysis in cancer cells. Bustamante and Pedersen [3] found a decreased inhibition of hexokinase by glucose-6-phosphate in rat hepatoma cells. Kahn et al. [4] described a glucose-6-phosphate dehydrogenase altered by some modifying factor. Meldolesi et al. [5] found a phosphofructokinase in rat thyroid tumor cells with a decreased ATP and citrate inhibition. The citrate inhibition could not be overcome by cyclic AMP.

Here we describe in addition the presence of a different hexokinase I. In leukemic lymphoblasts there is a decrease of the inhibition by glucose-1,6-diphosphate as can be seen by the difference in K_i values ($P < 0.05$). Besides this phenomenon a diminished influence of P_i on this inhibition is found. The inhibition of glucose-1,6-diphosphate is not abolished by the addition of P_i.

The presence of at least two subtypes of hexokinase type I with different regulatory properties was suggested for erythrocyte hexokinase [17] and confirmed by Vowles and Easterby [24] for hexokinase I from pig heart. Their results show that the activity of one form is overcome by P_i after glucose-6-phosphate inhibition more than the other. It was hypothesized by Rijksen and Staal [17] that a phosphate-insensitive form of hexokinase I (called type Ic) is formed out of a phosphate sensitive form (form Ib) by some post-synthetic modification. According to this hypothesis, it might be possible that the modification process in the leukemic CEM cells works at an elevated level in comparison to normal lymphocytes and non-leukemic lymphoblasts.

In agreement with a shift in hexokinase I subtypes is the electrophoretic

pattern of the hexokinase I subisozymes of lymphoid cells. In normal lymphocytes and stimulated lymphoblasts type Ib is predominant, whereas in the CEM cells this form is absent and type Ic predominates.

Further investigations are now directed to the question of which process is responsible for the interconversion of the different forms of hexokinase I.

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