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# Isozyme distribution of hexokinase, phosphofructokinase and pyruvate kinase in lymphocytes from patients with chronic lymphocytic leukemia

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## Summary

The enzyme activities and isozyme distribution of the three glycolytic regulator enzymes hexokinase, phosphofructokinase and pyruvate kinase were studied in lymphocytes of patients with chronic lymphocytic leukemia. Isozyme distribution patterns were determined by kinetic measurements, electrophoresis and immunoprecipitation.

The CLL lymphocytes were different from normal non-T lymphocytes with respect to hexokinase residual activity in the presence of glucose-1,6-$P_2$, pyruvate kinase residual activity in the presence of alanine, and phosphofructokinase activity after stimulation by glucose-1,6-$P_2$. No differences could be discerned in enzyme activities between the CLL and the normal T and non-T lymphocytes.

## Introduction

It is well known that tumor cells can differ from normal cells with respect to protein synthesis [1]. The observed protein patterns may be due to malignant transformation or may be similar to those found in an earlier differentiation stage of the normal cell. In the latter case the tumor cells should not be regarded as transformed but rather as blocked in maturation as is the case e.g. in the myeloid [2] and lymphocytic leukemias [3].

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Lymphocytes have been studied for cell surface markers [4], glycolipids [5], hormone receptors [6] and enzymes [7]. Amongst these enzymes terminal deoxynucleotidyl transferase, which serves as a marker for young lymphoblasts [8], is a well-known example. In addition, the purine enzymes adenosine deaminase, purine nucleoside phosphorylase and 5′-nucleotidase have been investigated in lymphocyte subpopulations and in leukemic lymphocytes (see [7] for a review). Adenosine deaminase has been reported to be high in these cells and may serve as a marker for B lymphocytes [10]. Lysosomal enzymes, e.g. hexosaminidase [11], acid phosphatase and α-naphthyl acetate esterase [12] may also serve as markers for lymphocyte subpopulations. Only a few reports concerning glycolytic enzymes are known. Lactate dehydrogenase has been studied in normal lymphocyte subpopulations [13,14] and in leukemic lymphocytes [15]. Meienhofer et al [16] and Vora [17] studied phosphofructokinase (ATP:D-fructose-6-P 1-phosphotransferase, PFK, EC 2.7.1.11) isozymes in human blood cells. We investigated the activities and the isoenzyme patterns of the glycolytic regulator enzymes hexokinase (ATP:D-hexose 6-phosphotransferase, HK, EC 2.7.1.1), phosphofructokinase and pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, PK, EC 2.7.1.40) in lymphocytes of patients with chronic lymphocytic leukemia (CLL).

## Materials and methods

### *Materials*

ATP, ADP, $NADP^+$, NADH, fructose-6-P (all as disodium salts), phosphoenolpyruvate (tricyclohexyl ammonium salt), glucose-1,6-$P_2$, fructose-1,6-$P_2$ (both as tetracyclohexylammonium 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 and bimethyl-thiazolyl-diphenol tetrazolium bromide 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 (gelatinized) was obtained from Chemetron (Milan, Italy).

### *Patients*

Patients with an established diagnosis of chronic lymphocytic leukemia were used in the study. Only patients with an absolute lymphocyte count of more than $20 \times 10^9$/l were admitted.

### *Sample preparation*

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, as described by Boyum [18]. This eliminated nearly all erythrocytes. Cells were used immediately or frozen as a dry pellet at −80°C. In the case of normal controls monocytes were removed by iron-carbonyl incubation for 1 h at 37°C and subsequent density centrifugation. This resulted in a lymphocyte suspension with less than 5% monocytes. Non-T lymphocytes were separated from T lymphocytes by sponta-

neous rosette formation with sheep erythrocytes treated with aminoethyl isothiouranium bromide (AET) and subsequent density centrifugation. This resulted in non-T lymphocytes with less than 5% T cells and virtually pure T lymphocytes. Cell lysis was performed by sonication for 30 s at 0°C with a 150 W ultrasonic desintegrator MK2 (MSE Scientific Instr., Crawley, UK). The extraction buffer for HK and PK 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 $\epsilon$-aminocaproic acid and 1 mmol/l di-isopropyl fluorophosphate. The extraction buffer for PFK in which 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 di-isopropyl fluorophosphate. The extraction buffer for the PFK immunoprecipitation was 0.05 mol/l Tris-phosphate buffer of 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 di-isopropyl fluorophosphate, 20 mmol/l $\epsilon$-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 were recovered from the pellet.

*Enzyme assays and kinetics*

HK activity was measured in the coupled glucose-6-phosphate dehydrogenase assay as described before [19]. PK activity was measured in the coupled lactate dehydrogenase assay as described by Bücher and Pfleiderer [20]. PFK 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, 1 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 U aldolase, 1.7 U glycerol-3-P dehydrogenase and 4.9 U triose-P isomerase. After 10 min preincubation of the sample at 37°C the reaction was started with 0.5 mmol/l $MgATP^{2-}$. All enzyme activities were expressed in units per mg protein, where 1 unit is defined as the amount of enzyme which catalyzes the formation of 1 $\mu$mol product per min at 37°C. The inhibition of HK by glucose-1,6-$P_2$ was determined at pH 7.15 and 37°C as described before [21]. Inhibition of PK by alanine was tested at pH 7.9 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 U 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. The influence of glucose-1,6-$P_2$ on PFK was studied at pH 7.4 and 37°C in 0.05 mol/l glycyl-glycine 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 U aldolase, 1.7 U glycerol-3-P dehydrogenase, 4.9 U triose-P isomerase and 0.5 mmol/l glucose-1,6-$P_2$. The auxiliary enzymes were extensively dialyzed at 4°C before use against 50 mmol/l glycyl-glycine, pH 7.4 containing 0.5 mmol/l $(NH_4)_2SO_4$ and 5 mmol/l dithiothreitol. After 20 min preincubation at 37°C the reaction was started by the addition of 0.5 mmol/l fructose-6-P. The reference value of 1.00 is the activity in the absence of glucose-1,6-$P_2$.

*Protein determination*

Protein content was measured according to the method described by Lowry et al [22] using bovine serum albumin as a standard.

*Electrophoresis*

HK electrophoresis on cellulose acetate at pH 8.8 was performed as described before [21]. Electrophoresis of PK was performed on cellulose acetate at pH 7.5 as was described elsewhere [23]. Selective staining of enzyme activities was performed [23]. After visualization of the enzyme bands the gels were scanned with a Helena Quickscan densitometer at 540 nm.

*Preparation of antibodies*

Preparation of antibodies against PFK M and F types was performed by immunization of New Zealand white rabbits by multiple subcutaneous injections of purified enzyme preparations from human muscle and human platelets as described by Cottreau et al [24]. The antimuscle PFK showed no cross-reactivity with type L and F PFK under the conditions of the immunoprecipitation assay. The purified platelet PFK consisted of F, L and a minor fraction of type M. The antiserum obtained against the platelet PFK was absorbed with partially purified human liver and purified human muscle PFK in excess. The enzyme remaining after absorption was removed from the immunoglobulins by DEAE cellulose chromatography 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 antiserum 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 according to the method described by Cottreau et al [24]. 4–5 mU of enzyme was 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 sera. 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 PFK activity in the supernatant was tested.

## Results

*Enzyme activities*

Table I summarizes the activities of HK, PFK and PK from CLL lymphocytes and normal controls, which were not different. The controls were normal peripheral blood lymphocytes depleted of monocytes. No differences were found between the enzyme activities of normal T, non-T lymphocytes or CLL lymphocytes.

TABLE I

ACTIVITIES OF HK, PK AND PFK FROM NORMAL AND CLL LYMPHOCYTES

| Enzyme | | Control lymphocytes | Control non-T lymphocytes | B-CLL |
|---|---|---|---|---|
| Hexokinase | (mU/mg) | 102 ±34 ($n=10$) | 81 ± 42 ($n=6$) | 76 ± 45 ($n=30$) |
| Pyruvate kinase | (U/mg) | 3.00± 1.14 ($n=10$) | 2.72± 1.77 ($n=5$) | 1.94± 1.18 ($n=30$) |
| Phosphofructokinase | (mU/mg) | 257 ±57 ($n=10$) | 230, 260 ($n=2$) | 324 ±158 ($n=30$) |

*Hexokinase isozyme distribution*

Four isozymes of HK are known, designated I to IV in order of their anodal electrophoretic mobility. The four isozymes differ in affinity for the substrate glucose and some inhibitors as glucose-6-P and glucose-1,6-$P_2$. The isozyme distribution was studied by means of electrophoresis and inhibition by glucose-1,6-$P_2$.

In Fig. 1 the scanning pattern of the HK electrophoresis from lymphocytes of two patients with CLL is shown. All patients' lymphocytes contained type I. In some cases besides the normal HK type I band a slower moving band in the type I region could be distinguished. In addition, HK type III seemed to be present in some patients, but the amount of the isozyme never exceeded 3% of total activity, as calculated by densitometry. Normal lymphocytes contain only HK type I (not shown in a Fig.).

Glucose-1,6-$P_2$ inhibition of HK at pH 7.15 and 37°C from normal and CLL lymphocytes is shown in Fig. 2. The values presented are the residual activities at glucose-1,6-$P_2$ concentration of 0.5 mmol/l. The residual activities in the CLL lymphocytes are significantly higher ($56 \pm 10$, $n = 29$) than in the normal lymphocytes ($42 \pm 4$, $n = 33$, $p < 0.001$).

*Pyruvate kinase isozyme distribution*

Three isozymes are known of PK designated L (liver) type, M (muscle) type and $M_2$ or K (kidney) type, which can be distinguished both by electrophoresis and kinetic analysis. The enzyme is a tetramer. Hybrid forms between the subunits are known to exist. The M and K isozymes differ markedly in their sensitivity to the inhibitor alanine; the M type is not affected, whereas the K type can be completely inhibited under appropriate conditions.

The densitometric analysis of the PK electrophoresis is given for lymphocytes from two CLL patients (Fig. 3). In all patients the $K_4$ isozyme and the $K_3M$ hybrid were seen in different ratios. In addition, some patients showed a minor $K_2M_2$ band (Fig. 3, patient a). Normal lymphocytes contain mainly $K_4$ and a little $K_3M$ hybrid (not shown in a Fig.).

Fig. 4 shows the comparison of the PK residual activity from normal and CLL lymphocytes after inhibition with alanine. The residual activities given are the values

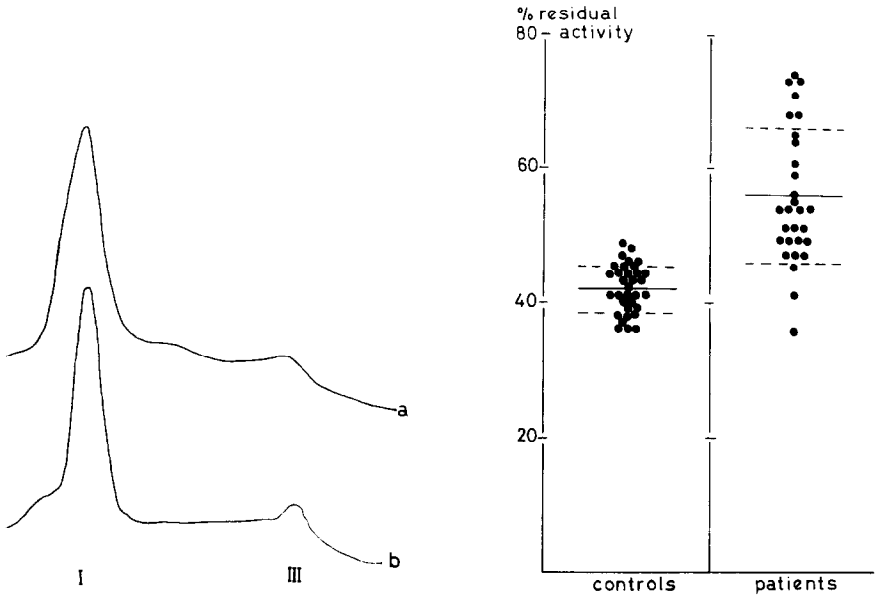


Fig. 1. Densitogram of HK electrophoresis from CLL lymphocytes from two patients (a and b).

Fig. 2. Residual activity of HK in the presence of 0.1 mmol/l glucose-1,6-$P_2$. The 100% value is the HK activity in the absence of glucose-1,6-$P_2$. Means±SD are indicated by bars and dotted lines, respectively. Controls: $n=33$, CLL lymphocytes $n=29$.

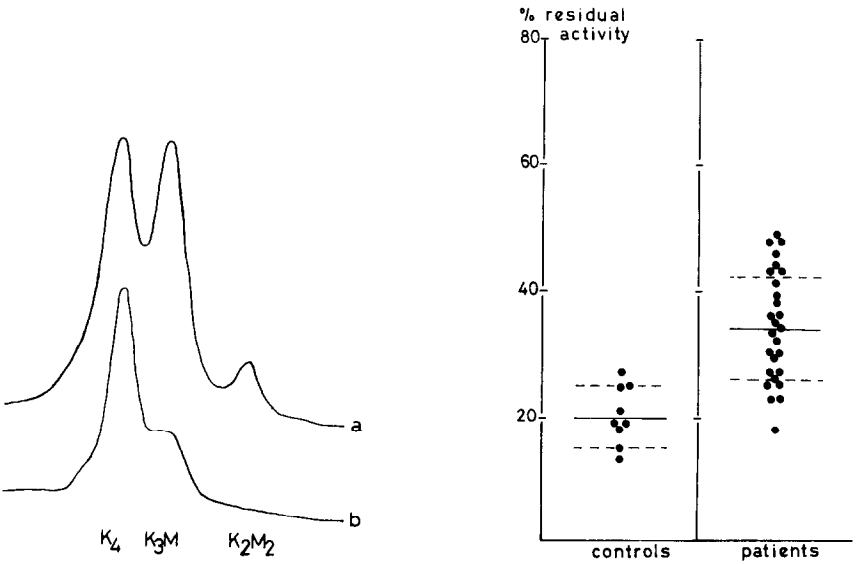


Fig. 3. Densitogram of PK electrophoresis from CLL lymphocytes from two patients (a and b).

Fig. 4. Residual activity of PK in the presence of 0.2 mmol/l alanine. The control group consists of non-T lymphocytes. The 100% value is the PK activity in the absence of alanine. Means±SD are indicated by bars and dotted lines, respectively. Controls: $n=9$, CLL lymphocytes: $n=27$.

at 0.2 mmol/l alanine at pH 7.8 and 37°C. The control lymphocytes have a significantly lower residual activity (20 ± 5%), than the CLL lymphocytes (34 ± 8%, $p < 0.005$).

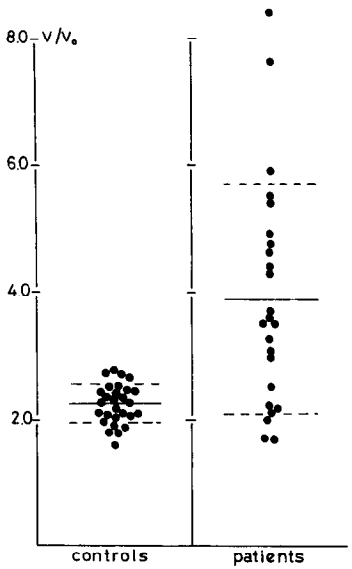


Fig. 5. Activation of PFK in the presence of 0.5 mmol/l glucose-1,6-$P_2$. V is the PFK activity in the presence of glucose-1,6-$P_2$, $V_0$ is the value in the absence of glucose-1,6-$P_2$. $V/V_0$ is the stimulation factor. Means ± SD are indicated by bars and dotted lines, respectively. Controls: $n = 28$, CLL lymphocytes: $n = 24$.

*Phosphofructokinase isozymes*

Three isozymes of PFK are known, designated as L (liver), M (muscle) and F (fibroblast) type. The enzyme exists as a tetramer. Hybrid forms of the several subunits do occur. The three isozymes differ as tested with kinetic measurements, e.g. glucose-1,6-$P_2$ is capable of stimulating the L and M type, whereas the F isozyme is insensitive to this compound [16].

The influence of glucose-1,6-$P_2$ on the enzyme activity in normal and CLL lymphocytes is shown in Fig. 5. The normal lymphocytes are moderately stimulated (activation factor 2.25 ± 0.30), compared to CLL lymphocytes (activation factor 3.90 ± 1.80, $p < 0.001$).

Residual activities after immunoprecipitation with excess of antibodies against PFK type L, M and F, respectively, are summarized in Fig. 6. Residual activities after neutralization with each of the three antibodies are of the same order in normal and patient lymphocytes. However, a greater heterogeneity is present in the CLL lymphocytes.

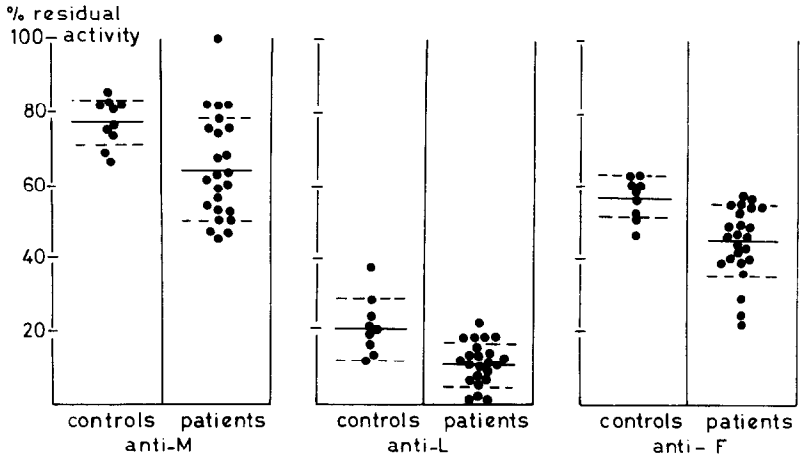


Fig. 6. PFK immunoprecipitation with anti-M (controls: $n=9$, CLL lymphocytes: $n=24$), anti-L (controls: $n=9$, CLL lymphocytes: $n=23$) and anti-F (controls $n=9$, CLL lymphocytes: $n=24$) from CLL lymphocytes. The 100% values are the PFK activities in the absence of antibody. Means±SD are indicated by bars and dotted lines, respectively.

## Discussion

Neoplasia is characterized by a misprogramming of protein synthesis in the tumor cells [25]. Evidence for this view is obtained from the observation that in many tumors the enzyme composition is altered in comparison to their normal counterparts. Regulator enzymes especially are involved [1]. With respect to alteration of regulator enzymes there are several possibilities. First a fully normal enzyme may be produced in larger amounts in a tumor in comparison with normal tissues. In that case only a rise in maximal activity is seen. Secondly, a decrease in maximal activity of an enzyme may be found. Thirdly a shift in isozyme distribution may occur (see [26] for a review). Fourthly, in tumors an enzyme may exist which has altered regulatory properties. Since, as is well known, intracellular glycolysis is normally regulated by the enzymes HK, PFK and PK, we may expect alterations of these enzymes in cancer cells. In this paper we deal with alterations of these enzymes in lymphocytes from patients with CLL in comparison with lymphocytes from peripheral blood of normal individuals.

With respect to HK isozyme distribution no change in isozyme pattern was observed after electrophoresis. However, marked differences in the affinity for the inhibitor glucose-1,6-$P_2$ between normal and CLL lymphocytes were found. It is well known that HK type I may bind to the outer membrane of mitochondria [27] and that the bound form of the enzyme is less inhibited by hexose-P inhibitors than the soluble form. It was suggested that a change in soluble particulate distribution could be a factor in regulation of HK activity in vivo [28]. The differences in HK I with respect to the inhibition by glucose-1,6-$P_2$ might be caused by alterations in the intracellular localization of the enzyme. The observed change in regulatory proper-

ties of hexokinase (glucose-1,6-$P_2$ inhibition) in CLL lymphocytes fits well with the hypothesis that regulator enzymes may be changed in tumor cells.

PK can exist in different forms: M, L and K type. In normal lymphocytes the L type is not expressed in contrast to the K isozyme, which is predominant; type M is a minor fraction. The K type is strongly inhibited by alanine, the M type not at all. So the more M subunits that are present, higher residual activity in the presence of alanine is found. From the results presented, we can conclude that CLL lymphocytes differ from normal lymphocytes with respect to the alanine inhibition of PK. In CLL lymphocytes higher residual activities were found. This means that in CLL lymphocytes the gene coding for the M subunits is activated more than in normal lymphocytes. Recently, Ibsen et al [29] reported that in the mouse system, only two genes are coding for the three pyruvate kinase isozymes: one gene for the K type and one for the L type. The M type would be formed from the K type by proteolysis. If the same phenomenon occurs in man, it would mean that the increased amounts of M subunits found in CLL lymphocytes could be explained by increased proteolytic conversion of K to the M type. No matter whether the higher residual activity shown here is due to activation of the gene coding for the M subunit or increased proteolysis, the difference found clearly shows that the mode of expression of PK has been altered in the CLL lymphocytes and this again fits well with the hypothesis that in tumor cells regulator enzymes are changed.

Three main types of PFK are known. The M, L and F types. Hybridization between the different subunits is known to occur. Glucose-1,6-$P_2$ is a positive effector of the L and M type, whereas the F type is not influenced by this ligand. In the presence of glucose-1,6-$P_2$ PFK from CLL lymphocytes is activated to a greater extent than the enzyme from normal lymphocytes. This difference may be due to a change in isozyme composition. We investigated therefore the isozyme pattern of PFK by immunoprecipitation with antibodies against the L, M and F type. We found only slight differences in phosphofructokinase isozyme distribution between normal and CLL lymphocytes, which may not completely explain the observed difference in glucose-1,6-$P_2$ influence. Yet, the enzyme in CLL lymphocytes is altered in regulatory properties.

It appears thus that all the three regulator enzymes of glycolysis from CLL lymphocytes show significant differences compared to the enzymes from normal non-T lymphocytes. This observation is in agreement with the hypothesis that regulator enzymes are changed in tumor cells. On the other hand the observed protein patterns in tumor cells may be similar to those found in an earlier differentiation stage of the normal cell, according to the 'block in ontogeny' theory.

In conclusion, significant differences were found for the three regulator enzymes of glycolysis between normal peripheral blood B lymphocytes and CLL B-cells. These findings may contribute to a better understanding of the lymphocyte metabolism in chronic lymphocytic leukemia.

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