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Control of Phosphofructokinase by Fructose 2,6-Bisphosphate in B-Lymphocytes and B-Chronic Lymphocytic Leukemia Cells[1]

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ABSTRACT

The levels of fructose 2,6-bisphosphate and glucose 1,6-bisphosphate and the activities of the key glycolytic enzymes have been studied in T- and B-lymphocytes, and in B-chronic lymphocytic leukemia cells (B-CLL). In both kinds of cells these two bisphosphorylated metabolites have been identified and are present at similar concentrations. Their phosphofructokinase, like that of other normal or tumoral cells, is sensitive to these activators. Fructose 2,6-bisphosphate is the most potent stimulator; it displays the properties of a positive effector. It greatly increases the affinity for fructose 6-phosphate and relieves the inhibition by adenosine triphosphate, without changing V_{max}. This effect is also synergistic with adenosine monophosphate. Despite few differences in the activity of phosphofructokinase and in the content of its main effectors in B-lymphocytes and in B-CLL cells, the kinetic properties of the enzyme from B-CLL cells were different, the enzyme being more sensitive to fructose 2,6-bisphosphate (K_a 2 orders of magnitude lower) and to glucose 1,6-bisphosphate than the enzyme from normal lymphocytes.

The results reported showing that phosphofructokinase from B-CLL lymphocytes is altered in regulatory properties and the observed changes, in comparison to phosphofructokinase from normal B-lymphocytes, fit well with the hypothesis that fructose 2,6-bisphosphate can also assume a regulatory role in these cancer cells characterized by proliferation and accumulation of relatively mature-appearing lymphocytes.

INTRODUCTION

It has been recognized for many years that glucose is an important metabolic fuel for tumors, particularly for poorly differentiated and rapidly growing tumors, that exhibit, unlike normal cells, high glycolytic rate under aerobic conditions (1–4). The biochemical mechanism of this phenomenon is not well understood.

The discovery of Fru-2,6-P_2[3] and the elucidation of its key role in the activation of PFK opened a new way of research in both normal cells and their neoplastic counterpart. Fru-2,6-P_2 is widely distributed in eukaryotic cells and is the most potent stimulator of PFK, one of the rate-limiting enzymes of glycolysis. Evidence has been presented for its involvement in the control of glycolysis by glucose and hormones in normal (5–9) and in cancer cells (10–15).

B-CLL is a disease characterized by the clonal proliferation of B-lymphocytes which maintain a relatively normal morphology (16). Several studies have demonstrated that B-CLL lymphocytes consume less total sugar and share an impaired carbohydrate metabolism (17, 18). In order to understand this observation, in contrast to the majority of neoplastic cells, we have investigated the activity of the key glycolytic enzymes and the concentration and the kinetic effects of two effectors of PFK (Fru-2,6-P_2 and Glu-1,6-P_2) in both normal peripheral blood lymphocytes and B-CLL cells.

MATERIALS AND METHODS

Enzymes and Chemical Reagents. Enzymes and chemical reagents were obtained from Boehringer Mannheim (Mannheim, Federal Republic of Germany) or from Sigma Chemical Co. (St. Louis, MO). Ficoll 400 and Sephadex G-25 Fine were from Pharmacia Fine Chemical (Uppsala, Sweden). Leuko-Pack Leukocyte filter was from Fenwal Lab (Thetford, England). RPMI 1640 and fetal horse serum were from Gibco (Paisley, Scotland).

Preparation of Pure Cell Suspensions and Cell Extracts. Lymphocytes from normal controls and from B-CLL were obtained from the patient populations attending the Hematology Department of the Hospital Clinic i Provincial de Barcelona. Heparinized and defibrinated peripheral blood samples (20–400 ml) were freshly obtained, the lymphocytes being separated from other blood cells by centrifugation in Ficoll (ρ = 1.077) at 700 × g for 20 min at 25°C. The lymphocytes were carefully collected from the interface, washed by resuspending in phosphate-buffered saline, twice, and recentrifuged according to the method of Boyum (19). Contaminant erythrocytes were removed by lysing cell pellets in the presence of 0.15 M NH_4Cl for 20 min. B- and T-lymphocytes from normal blood samples were separated and B-lymphocytes were purified by the nylon wool column filtration method of Greaves and Brown (20). All the cells were resuspended in 50 mM phosphate buffer (pH 6.4), containing 0.2 mM NADP+, 20 mM 6-aminohexanoic acid, 20 mM EDTA, and 3 mM 2-mercaptoethanol. The volume of lymphocytes was obtained by sizing the cells, using a Coulter Channalyzer system, and multiplying their number.

Enzyme Assays. Fresh or frozen cells (20 × 10^6 cells/ml) were disrupted by freeze-thawing and centrifuged (10,000 × g for 10 min), and appropriate dilutions of the supernatant were used for enzyme assays. The activities of PFK (EC 2.7.1.11), PK (EC 2.7.1.40), and HK (EC 2.7.1.1) were measured under V_{max} conditions (21). The assays were carried out at 37°C in a final volume of 1 ml in a thermostated recording spectrophotometer Beckman Model 25 Kinetic system. No significant differences in enzyme activities were observed between fresh and frozen cells.

Partial Purification of PFK. For the partial purification of PFK, fresh pure cells from normal and B-CLL were disrupted by freeze-thawing. After addition of 0.1 mM fructose 6-P, 0.3 mM glucose 6-P and 50 μM Fru 1,6-P_2, they were centrifuged (15,000 × g for 15 min); the enzyme present in the supernatant was precipitated by 60% ammonium sulfate and collected by centrifugation (15,000 × g for 15 min); and the pellet was resuspended in 20 mM K_2HPO_4, 50 mM NaF, 1 mM fructose 6-P 20 μM EDTA, and 5 mM 2-mercaptoethanol at pH 7.6. The solution was layered into a column (2 × 20 cm) of Sephadex G-25 Fine equilibrated with the same buffer and the peak fractions were pooled and used for the kinetic measurements. All the steps were performed at 0–4°C.

Kinetic Studies of PFK. Kinetic studies were performed at 30°C in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.1) containing 100 mM KCl, 1 mM NH_4Cl, 5 mM $MgCl_2$, triosephosphate isomerase (5 units/ml), glycerol 3-phosphate dehydrogenase (1.7 units/ml), phosphoglucose isomerase (0.70 unit/ml), aldolase (0.5 unit/ml), 0.15 mM NADH, and the indicated concentrations of MgATP, fructose 6-P and other effectors of PFK. Reactions were started with

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[3] The abbreviations used are: Fru-2,6-P_2, fructose 2,6-bisphosphate; B-CLL, chronic lymphocytic leukemia type B; Glu-1,6-P_2, glucose 1,6-bisphosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; PBS, phosphate-buffered saline, pH 7.2; PFK, phosphofructokinase; PK, pyruvate kinase; HK, hexokinase; BSA, bovine serum albumin.

fructose 6-P. All the auxiliary enzymes used were previously desalted through a Sephadex G-25 Fine column (22).

Analytical Methods. Assay for Fru 2,6-P_2 was performed according to the method of Van Schaftingen *et al.* (23), using the stimulation of potato tuber pyrophosphate:fructose 6-P phosphotransferase by fructose 2,6-P_2. Glucose 1,6-P_2 was assayed spectrophotometrically as a cofactor of phosphoglucomutase (24). Protein concentration in the solution was measured according to the method of Lowry *et al.* (25) using bovine serum albumin as a standard.

RESULTS

Purity of Isolated Cell Suspensions. Lymphocyte cell suspensions were generally 95% pure, monocytes being the occasional contaminants. Lymphocytes B and T isolated from peripheral blood were ≥85% pure; the contaminants were lymphocytes T and B, respectively, and monocytes. Lymphocytes obtained from peripheral blood specimens of patients with chronic lymphocytic leukemia showed a high degree of purity (≥98%). The use of defibrinated blood resulted in negligible contamination with platelets.

Lymphocyte Enzyme Activities. The activity levels of PFK, PK, and HK, expressed as units/10^9 cells and in units/ml of cells, in both normal peripheral blood lymphocytes (T and B) and in lymphocytes of B-CLL are shown in Table 1. When compared with normal B-lymphocytes, B-CLL cells showed lower values for all enzyme activities when expressed in units/10^9 cells. However, if the activities were expressed in units/ml of cells, only the HK showed statistically significant decreased activities ($P < 0.001$).

Levels of Bisphosphorylated Metabolites. Fru-2,6-P_2 and Glu-1,6-P_2 content of normal lymphocytes and B-CLL cells are shown in Table 2. In normal lymphocytes the levels of both metabolites were higher in B- than in T-cells. Compared to normal B-lymphocytes, Fru-2,6-P_2 content of B-CLL was lower whereas Glu-1,6-P_2 showed similar values.

Sensitivity of PFK to Bisphosphorylated Metabolites. In both kinds of cells (normal B-lymphocytes and B-CLL cells), Fru-2,6-P_2 displayed the typical properties of a positive effector of PFK. It greatly increased the affinity of PFK for fructose 6-P (Fig. 1*A*) and decreased the inhibitory effect of ATP (Fig. 1*B*) without changes in V_{max}. Their apparent $S_{0.5}$ for fructose 6-P in the absence of Fru-2,6-P_2 was 0.8 mM in B-CLL and 0.6 mM in normal B-lymphocytes (Fig. 1*A*), but in the presence of Fru-2,6-P_2, the $S_{0.5}$ for fructose 6-P decreased in B-CLL more than in normal B-lymphocytes. Furthermore, the inhibitory effect of high concentrations of ATP in presence of Fru-2,6-P_2 was less important in B-CLL than in normal B-lymphocytes (Fig. 1*B*). The study of synergism between Fru-2,6-P_2 and AMP, at physiological concentrations of ATP (2.5 mM) and fructose 6-P (50 μM), showed that in normal B-lymphocytes AMP or Fru-2,6-P_2 alone were not able to stimulate normal PFK, whereas a stimulatory effect was obtained when both metabolites were added together. Conversely, in B-CLL, PFK activity was strongly stimulated by Fru-2,6-P_2 even in the absence of AMP (Fig. 2).

The stimulatory effect of hexose bisphosphates on PFK activity from both normal B-lymphocytes and B-CLL cells is shown in Fig. 3. Although both PFKs were activated by Fru-2,6-P_2 and Glu-1,6-P_2, PFK from B-CLL was activated to a greater extent than PFK from normal lymphocytes. Thus, half-maximal stimulation effect of hexose bisphosphates on B-CLL-PFK was obtained at 0.01 μM Fru-2,6-P_2 (4 μM for normal B-cells) and at 6 μM Glu-1,6-P_2 (60 μM for normal B-lymphocytes). Inhibitory effects of Glu-1,6-P_2 at millimolar concentrations are also apparent in both cases.

DISCUSSION

B-CLL is a lymphoid neoplastic disease characterized by proliferation and accumulation of relatively mature-appearing lymphocytes (16). B-CLL cells compared to normal lymphocytes consume less total sugar and metabolize less carbohydrate via the hexose monophosphate shunt pathway leading to glycogen accumulation (18), manifested by increasing quantities of intracytoplasmatic material stainable with the periodic acid-Schiff reagent (26). This behavior is contrary to the majority of the cancer cells with metabolism that is characterized by the predominance of aerobic glycolysis (1–4). Previous studies using leukemia lymphoid cells have shown that several membrane or cytosolic enzymes exhibit changes in total activity and qualitative isozymic shifts that may be used as cellular markers. This is especially true for the purine pathway enzymes adenosine deaminase, nucleoside phosphorylase, and 5′-nucleotidase (27–29). We have studied the total activities of the key glycolytic enzymes (PFK, PK, and HK) in B-CLL lymphocytes and our results demonstrated that, compared to purified normal B-lymphocytes, the activity of PFK, PK, and HK from B-CLL extracts is lower when expressed as units/10^9 cells and the activity of PFK and PK is normal and that of HK is lower when expressed as units/ml of cells. These differences in the expression of the results are probably due to the decreased volume of B-CLL cells [162.2 ± 16.3 (SD) fl], compared to B-lymphocytes (224.7 ± 11.0 fl). The significant decrease in the hexokinase activity in B-CLL cells, in addition to the altered kinetic properties of HK, PFK, and PK (30), could explain the impaired carbohydrate metabolism described previously (17, 18).

We have shown that Fru-2,6-P_2 and Glu-1,6-P_2 are present in normal and B-CLL cells at similar concentrations and that their PFK, like that of other normal (5–9) or tumoral (10–15) cells, is sensitive to these stimulators. Fru-2,6-P_2 is the most potent stimulator of PFK in these cells, increases its apparent affinity for fructose 6-P, and decreases the inhibitory effect of ATP, without changing V_{max} (Fig. 1). This effect is also synergistic with AMP (Fig. 2). However, some differences between normal and B-CLL lymphocytes were herein observed. (*a*) The two kinds of cells showed different K_a for Fru-2,6-P_2, lympho-

Table 1 *Activities of phosphofructokinase, pyruvate kinase, and hexokinase from normal lymphocytes and lymphocytes from B-CLL*

	PFK		PK		HK	
Cell type	Units/10^9 cells	Units/ml	Units/10^9 cells	Units/ml	Units/10^9 cells	Units/ml
Total lymphocytes	1.3 ± 0.5[a] (9)[b]	5.7 ± 2.6 (6)	41.0 ± 10.1 (8)	153.1 ± 69.8 (6)	2.4 ± 0.9 (10)	11.8 ± 3.1 (6)
T-Lymphocytes	1.5 ± 0.3 (6)	8.5 ± 1.5 (6)	33.0 ± 6.3 (6)	187.5 ± 36.4 (6)	2.0 ± 0.2 (6)	11.3 ± 1.1 (6)
B-Lymphocytes	1.8 ± 0.6 (9)	6.0 ± 1.3 (5)	78.7 ± 32.6 (9)	248.1 ± 55.1 (5)	4.5 ± 2.1 (11)	17.5 ± 5.4 (6)
B-CLL	1.1 ± 0.6 (11)[c]	7.8 ± 3.6 (11)	26.2 ± 11.3 (10)[c]	162.7 ± 80.9 (10)	1.1 ± 0.6 (11)[c]	6.4 ± 3.3 (11)[c]

[a] Mean ± SD.
[b] Numbers in parentheses, number of separate donors.
[c] Significantly different ($P < 0.001$ by paired *t* test) *versus* control B-lymphocytes.

Table 2 *Levels of bisphosphorylated metabolites in normal lymphocytes and lymphocytes from patients with B-CLL*

Cell type	Fru-2,6-P_2		Glu-1,6-P_2	
	nmol/10^9 cells	nmol/ml	nmol/10^9 cells	nmol/ml
Normal lymphocytes	1.1 ± 0.5[a] (14)[b]	5.7 ± 1.8 (6)	1.8 ± 0.9 (11)	8.6 ± 3.3 (6)
T-Lymphocytes	1.1 ± 0.3 (14)	6.2 ± 1.2 (6)	0.8 ± 0.4 (8)	10.2 ± 3.9 (6)
B-Lymphocytes	2.5 ± 0.6 (7)	11.1 ± 2.0 (5)	2.7 ± 0.6 (6)	12.0 ± 2.0 (5)
B-CLL	0.8 ± 0.4 (8)[c]	4.9 ± 1.8 (8)[c]	2.5 ± 1.6 (8)	15.4 ± 7.5 (8)

[a] Mean ± SD.
[b] Numbers in parentheses, number of separate donors.
[c] Significantly different ($P < 0.001$ by paired t test) *versus* control B-lymphocytes.

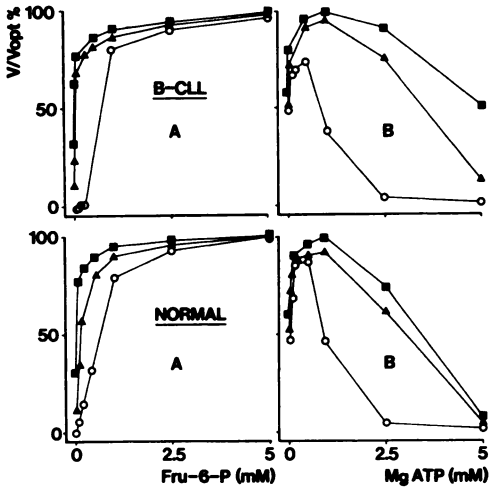

Fig. 1. Effect of Fru-2,6-P_2 on the affinity of PFK for fructose 6-P (*A*) and the inhibition of the enzyme by ATP (*B*). All assays were performed in the presence of 5 mM P_i and 0.1 mM AMP. In *A*, ATP concentration was 1.5 mM; in *B*, fructose 6-P concentration was 0.25 mM. Fru-2,6-P_2 concentrations: ○, none; ▲, 1 μM; ■, 5 μM. Values are expressed as percentages of the optimal velocities (*Vopt*). Similar data were obtained in two other experiments.

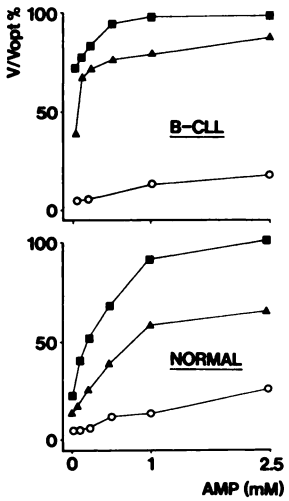

Fig. 2. Effect of AMP on the activity of the PFK measured at various concentrations of Fru-2,6-P_2. All assays were performed in the presence of 5 mM P_i, 2.5 mM ATP, and 50 μM fructose 6-P. Fru-2,6-P_2 concentrations: ○, none; ▲, 1 μM; ■, 5 μM. Values are expressed as percentages of the optimal velocities (*Vopt*). The values shown are means of two experiments.

cytes of B-CLL being more sensitive to the activating effect (2 orders of magnitude) than normal B-lymphocytes. With respect to Glu-1,6-P_2, another positive effector of PFK, the results obtained are similar to those reported by Kraaijenhagen *et al.* (30), showing that PFK from B-CLL cells was activated to a

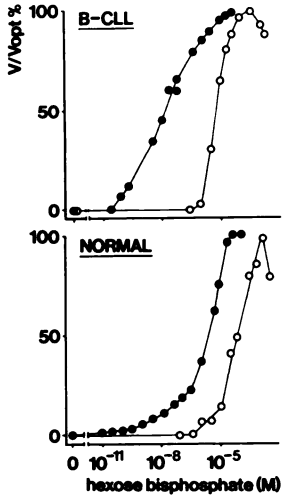

Fig. 3. Stimulation of PFK by hexose bisphosphates. The assays were performed in the presence of 1 mM AMP, 0.2 mM P_i, 1.5 mM ATP, and 0.5 mM fructose 6-P. Fru-2,6-P_2 (●); glu-1,6-P_2 (○). Values are expressed as percentages of the optimal velocities (*Vopt*). Similar results were obtained in other two experiments under the same conditions.

greater extent (K_a 1 order of magnitude lower) than the enzyme from normal lymphocytes. The PFK from B-lymphocytes and B-CLL cells were inhibited at a high concentration of Glu-1,6-P_2, probably by product inhibition competing with Fru-1,6-P_2 (31) (Fig. 3). (*b*) The response of PFK from normal cells to Fru-2,6-P_2 was greatly dependent on the concentration of AMP. However, B-CLL cells showed a more synergistic effect in the presence of both effectors, the K_a for AMP in these conditions being inferior to normal cells.

Similar abnormal kinetic patterns of PFK in leukemic cells have been reported showing a less inhibitory effect of ATP in nonlymphoblastic leukemic cells (32) and a more stimulatory effect of Glu-1,6-P_2 in B-CLL lymphocytes (30). These results as well as ours suggest that the observed kinetic differences can be due to changes in isoenzymatic pattern or to differential phosphorylation states of PFK. The hypothesis of a shift in subunit composition of the enzyme during neoplastic transformation has been already shown (14, 33–35). This change involves the predominance of platelet and liver-type subunits upon muscle-type subunits in the enzyme structure (14). Moreover, it has been demonstrated that the desensitization of the enzyme to potent inhibitors (ATP and citrate) and increased sensitivity to the activator Fru-2,6-P_2 (14) could probably reflect a predominance of the liver-type subunit with higher affinity for Fru-2,6-P_2 than muscle-type subunits (34). This hypothesis has been clearly demonstrated for several neoplastic cells (14, 35), but not for B-CLL lymphocytes (35).

Another hypothesis for explaining the kinetic modifications of PFK observed in B-CLL cells could be the covalent modification of the enzyme. Although the role of phosphorylation in modulating kinetics of PFK has been questioned (36), this possibility cannot be ruled out. In this way, Kuratsune *et al.* (37) have shown that endogen proteins of B-CLL lymphocytes are more strongly phosphorylated than in other leukemic cells. These differences in the phosphorylation estate of endogenous proteins may be related to the activation of different protein kinases (2). Several tumor promoters have the property of activating protein kinase C both *in vitro* and *in vivo* (38) and the activation of this enzyme could explain the altered kinetic properties of PFK, similar to the kinetic changes described by the rabbit muscle enzyme (39).

Further exploration of the role of the different protein kinases

and their effects on the regulatory enzymes of metabolic pathways may allow an understanding of the biochemical basis of the pleiotropic changes in cancer cells.

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