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Metabolism of 6-Mercaptopurine in Human Leukemic Cells[1]

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SUMMARY

The PRPP concentrations, PRPP formation, and phosphorylation of 6-mercaptopurine in leukocyte suspensions and homogenates prepared from leukemic patients were studied. The rate of phosphorylation was 3 orders of magnitude higher in leukocyte homogenates (329 ± 56 nmoles/3 × 10^7 cells/hr) than in leukocyte suspensions (0.01 to 0.52 nmole/3 × 10^7 cells/hr). Leukocytes contained less than 0.03 nmole PRPP/3 × 10^7 cells, which was less than the amount of thioinosinic acid formed in the leukocyte suspensions. Therefore, PRPP must be synthesized during the incubation. The amount of PRPP formed in leukocytes from 10 patients with acute leukemia varied from 5.2 to 38.0 nmoles/3 × 10^7 cells/hr. The cells which had higher PRPP formation also had higher thioinosinic acid formation. Thus, the availability of PRPP may play a key role in thioinosinic acid formation in human leukemic leukocytes.

INTRODUCTION

6-MP[2] must be converted to TIMP to be biologically active (1-3). This conversion is catalyzed by HGPRTase and requires PRPP (1, 3). The predominant mechanism of resistance to 6-MP in experimental animal tumors and bacteria is the loss of HGPRTase (1, 3, 4). On the other hand, nucleotide synthesis from purine bases is limited by the concentration of PRPP (5). This suggests that the PRPP content of cells may affect TIMP formation and contribute significantly to the clinical effect of 6-MP. In order to clarify the role of PRPP in the conversion of 6-MP to TIMP, we examined HGPRTase activity, PRPP concentration, and PRPP formation in human leukemic leukocytes.

MATERIALS AND METHODS

Chemicals. All materials used were obtained from Sigma Chemical Co., St. Louis Mo. [8-^{14}C]-6-MP (2.5 mCi/mmole), [8-^{14}C]HX (20.7 mCi/mmole), [8-^{14}C]inosine (40.0 mCi/mmole), and L-[*U*-^{14}C]glutamine (30 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England.

Isolation of Erythrocytes. Blood was drawn by venipuncture into heparinized tubes. The plasma and the buffy coat were separated by centrifugation in the cold and removed. Sedimented erythrocytes were washed twice with cold 0.9% NaCl solution.

Isolation of Leukocytes. Leukocytes were isolated from peripheral blood by the dextran sedimentation procedure (18). Contaminating erythrocytes were lysed by adding 3 volumes of water at 0°. After 40 sec, isotonicity was restored by addition of 1 volume of 3.6% NaCl solution. The unbroken cells were collected by centrifugation at 500 × *g* for 10 min and washed twice with 0.9% NaCl solution by resuspension and centrifugation.

Isolation of L1210 Cells. L1210 cells, harvested from mice on the 7th day after i.p. inoculation of 3 × 10^6 cells, were washed twice with 0.9% NaCl solution. Erythrocytes were removed by lysis with hypotonic NaCl solution as described above.

Determination of TIMP Formation. The leukocytes were resuspended in Eagle's minimum essential medium containing 30% calf serum in a concentration of 3 × 10^7 cells/ml. The leukocyte suspension was incubated at 37° for 60 min in the presence of 0.05 μmole of [8-^{14}C]-6-MP. At the end of the incubation, the mixture was chilled in an ice bath, and the cells were collected by centrifugation and washed twice with 1 ml of Eagles's minimum essential medium. The leukocytes were resuspended in 1 ml of Eagle's minimum essential medium, and the acid-soluble fraction was obtained by addition of 0.05 ml of 10 M perchloric acid. The residue was washed again with 1 ml of 0.5 M perchloric acid. The extracts were combined and neutralized with potassium hydroxide. The neutralized extracts were chromatographed on a 1 x 20-cm column of Dowex 1-formate resin with the appropriate carriers, using a formic acid gradient elution system (0-12N). Column eluates were collected in 0.5-ml increments with an automatic fraction collector. Nucleotides formed were determined by measuring the radioactivity of each fraction and are presented in terms of percentage of total radioactivity incorporated into the cells during the incubation.

Extraction of PRPP. The sedimented leukocytes or L1210 cells, prepared above, were suspended in 1 mM EDTA, pH 7.4, at a concentration of 2 × 10^7 cells/ml. The suspensions were deproteinized by heating in boiling water for 2 min and then were promptly chilled in an ice bath. The denatured protein was sedimented by centrifugation, and the clear supernatant fluid was used immediately for assay of the PRPP content. The recovery test showed that EDTA completely prevented the decomposition of PRPP during the 2 min of heating.

Assay of PRPP. The PRPP content of leukocytes and

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[2] The abbreviations used are: 6-MP, 6-mercaptopurine; TIMP, thioinosinic acid; HGPRTase, hypoxanthine guanine phosphoribosyltransferase; PRPP, phosphoribosylpyrophosphate; HX, hypoxanthine; PRPPATase, phosphoribosylpyrophosphate-amidotransferase.

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L1210 cells was determined by a modification of the procedure described by Henderson and Khoo (6). The reaction mixture contained 55 μmoles Tris buffer, pH 7.4; 5 μmoles $MgCl_2$; 10 nmoles [8-^{14}C]HX; 0.09 ml of the cell extract, and 0.1 ml of hemolysates in a final volume of 0.25 ml. After 20 min incubation at 37°, the reaction was stopped by addition of 2 μmoles neutralized EDTA, and an aliquot (20 μl) of the reaction mixture was spotted on Toyo No. 51 paper with appropriate carriers. IMP produced was separated from the substrate by high-voltage electrophoresis using 0.05 M borate buffer, pH 9.0, containing 0.001 M EDTA at 4000 volts for 20 min (R_F values were: HX, 0.30; IMP, 1.00). The radioactivities were determined by cutting the chromatographic paper into 0.5-cm strips and counting them in toluene-based scintillation fluid by a Packard Tri-Carb scintillation counter. The amounts of PRPP in the assay medium were calculated from the IMP produced. The standard curves showed a linear responsiveness up to 5 nmoles of PRPP.

Enzyme Preparations. Erythrocytes were hemolyzed by adding 1 ml of 0.2 M Tris buffer, pH 7.4, to 2 × 10^9 packed cells, and the hemolysates were used as crude HGPRTase for the assay of PRPP. Leukocytes (3 × 10^7 cells) were suspended in 0.1 ml of 0.001 M Tris buffer, pH 7.4, and were homogenized by a Teflon homogenizer for 40 sec. Homogenates were used for determination of HGPRTase activity. L1210 cells or leukocytes were suspended in 1 volume of 0.2 M Tris buffer, pH 7.4, and homogenated for 40 sec with a Teflon homogenizer. After centrifugation at 10,000 × *g* for 10 min, the supernatant was used for the assay of PRPPATase (19). L1210 cells (2 × 10^6 cells) were suspended in 1 ml of ice-cold, distilled water and homogenized for 40 sec with a Teflon homogenizer. The homogenates were used for the assay of inosine phosphorylase (12).

Enzyme Assay. HGPRTase activity was assayed by the method of Kelley *et al.* (11). The final reaction mixture (100 μl) contained 5.5 μmoles Tris buffer, pH 7.4; 0.5 μmole $MgCl_2$; 0.1 μmole PRPP; 0.05 μmole [8-^{14}C]-6-MP or 0.05 μmole [8-^{14}C]HX; and homogenates of 2 × 10^6 leukocytes. After incubation for 10 min at 37°, the reaction was terminated by the addition of 2 μmoles neutralized EDTA.

An aliquot (20 μl) of the reaction mixture was spotted on Toyo No. 51 paper with the appropriate carriers. Nucleotides produced were separated from the substrate, and the radioactivities of the substrate and the separated nucleotides were determined under the same conditions as those described in the assay of PRPP (R_F values were: 6-MP, 0.45; TIMP, 1.0). Nucleotide formation was shown to have a linear relationship with incubation time and cell number under the conditions used. PRPPATase was assayed by the radiochemical method of Tay *et al.* (19). The reaction mixture contained 55 μmoles Tris buffer, pH 7.4; 5 μmoles $MgCl_2$; 100 nmoles L-[*U*-^{14}C]glutamine; 30 nmoles PRPP; 2.5 μmoles dithiothreitol; and 1 to 2 mg protein in a final volume of 0.25 ml. After 20 min incubation at 37°, the reaction was stopped by adding 2 μmoles neutralized EDTA. An aliquot (20 μl) of the reaction mixture was spotted on Toyo No. 51 paper with appropriate carriers and was subjected to electrophoresis under the same conditions as those described in the assay of PRPP. The chromatographic paper was stained with acetone containing 0.1% ninhydrin-0.5% acetic acid. Glutamine and glutamate were found to be completely separated (R_F values were: glutamine, 0.20; glutamate, 1.0). The radioactivities of the substrate and the separated glutamate were determined under the same conditions as those described in the assay of PRPP. PRPPATase activity was calculated from the ratio of the radioactivities of glutamine to glutamate. Glutamate formation showed a linear relationship to incubation time and protein concentration under the conditions used.

Assay of inosine phosphorylase activity was based on the method described by Kim *et al.* (12). The reaction mixture contained 50 μmoles potassium phosphate buffer, pH 7.4; 0.5 μmole [8-^{14}C]inosine; and homogenates of 1 × 10^5 to 2 × 10^5 cells in a final volume of 1 ml. After 10 min incubation at 37°, the reaction was stopped by heating in boiling water for 2 min. The denatured protein was deposited by centrifugation, and aliquots (20 μl) of the supernatant were examined by paper chromatography. The R_F values of inosine and HX with 5% disodium hydrogen phosphate as eluent were 0.66 and 0.46, respectively; using 44% aqueous propionic acid-93.8% aqueous *n*-butyl alcohol (1:1) as eluent, the R_F values were 0.48 and 0.70, respectively. HX formation showed a linear relationship under the conditions used. Protein was measured by the method of Lowry *et al.* (13).

RESULTS

Since conversion of 6-MP to TIMP is believed to be required for the biological action of 6-MP (1, 3), we have attempted to ascertain the extent of this conversion in leukemic cells. Column chromatography of the acid-soluble fraction of leukocytes incubated with [8-^{14}C]-6-MP indicated 5 principal radioactive peaks. Four of the peaks corresponded to HX, 6-MP, thioxanthine, and TIMP by exact coincidence of the radioactive peak with the appropriate carrier. The 5th peak, which eluted after TIMP, has not been characterized. Radioactivities detectable in HX, 6-MP, thioxanthine, TIMP, and the unknown peak were 2.7, 45.6, 6.3, 38.5, and 6.5% of the total radioactivity found in the leukocyte fraction, respectively. These data indicate that 10.4% (0.52 nmole) of the 6-MP (0.05 μmole) added to the medium was converted to TIMP (Chart 1). TIMP formation in

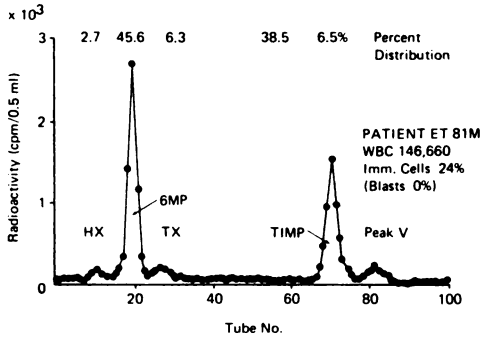

Chart 1. Dowex 1 column chromatogram of the acid-soluble fraction of leukocytes incubated with [8-^{14}C]-6-MP *in vitro*. [8-^{14}C]-6-MP (0.05 μmole) was incubated for 60 min with leukocyte suspensions (3 × 10^7), and neutralized perchloric acid extracts were then chromatographed as described in "Materials and Methods."

leukocytes from 10 patients (2 patients with acute myeloblastic leukemia and 8 patients with chronic myelogenous leukemia) varied from 0.01 to 0.52 nmole/3 × 10^7 cells/hr (Chart 2).

Attention was devoted to investigating the relationship between the inability of leukocytes to synthesize TIMP, especially the enzyme responsible for this synthesis, and the supply of PRPP in leukocytes. HGPRTase activity for 6-MP, obtained for 7 patients, was 329 ± 56 nmoles/3 × 10^7 cells/hr, and HGPRTase activity was 765 ± 77 nmoles/3 × 10^7 cells/hr (mean ± S.D.). All 7 patients examined showed similar enzyme activity. Using the radiochemical assay, it is possible to detect amounts of PRPP as low as 0.03 nmole/3 × 10^7 cells in leukocytes. However, no significant formation of [8-^{14}C]IMP was observed when [8-^{14}C]HX was incubated with human leukocytes. In contrast, considerable PRPP (4.9 nmoles/3 × 10^7 cells) was found in L1210 cells. This PRPP concentration is similar to that found for Ehrlich ascites carcinoma cells (6, 14).

Our previous study (8) showed that the K_m for PRPP in *in vitro* phosphorylation, where HX and PRPP were used as the substrates, was 2.0 × 10^{-4} M. The amount of PRPP present in human leukocytes, however, is far less than that required for TIMP formation in leukocyte suspensions. It is, therefore, likely that the intracellular concentration of PRPP may be an important factor in regulation of TIMP synthesis in leukemic leukocytes.

Even after suspending leukocytes in Eagle's minimum essential medium at 37° for 60 min, the intracellular concentrations of PRPP did not increase to the threshold of the assay. Since Hershko *et al.* (7) reported that PRPP formation was enhanced by P_i, we examined the effects of P_i on PRPP formation in leukocyte suspensions. After 1 hr incubation, in the presence of 60 mM P_i, there was a marked elevation of the concentration of PRPP. The amount of PRPP formed in leukocytes from 10 patients with acute leukemia varied from 5.2 to 38.0 nmoles/3 × 10^7 cells/hr (Chart 3).

The relationship between the PRPP formation in leukocytes and percentage of immature leukocytes is shown in Chart 4*A*. A significant negative correlation was obtained between the PRPP formation and percentage of immature cells ($t = -0.91$; $p < 0.01$). In other patients, the TIMP formation also appeared to be relatively higher in mature

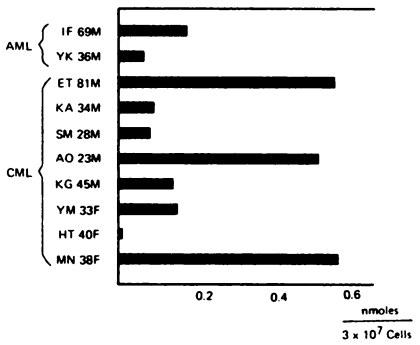

Chart 2. Variation of TIMP formation in leukocytes obtained from patients with leukemia. The formation of TIMP was determined under the same conditions as described in the legend of Chart 1. *AML*, acute myeloblastic leukemia; *CML*, chronic myelogenous leukemia.

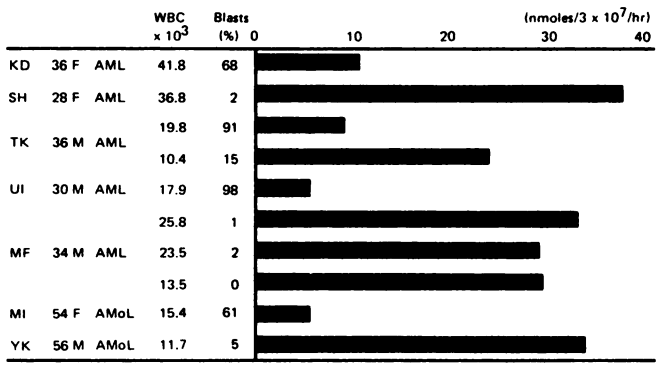

Chart 3. The PRPP formation in human leukemic leukocytes. Leukocytes (1 × 10^7) from patients with acute myeloblastic leukemia (*AML*) or acute myelomonocytic leukemia (*AMoL*) were suspended in 1 ml of 0.9% NaCl solution containing 10 mM glucose and 60 mM P_i. The suspensions were incubated for 60 min at 37°. The PRPP concentrations were determined as described in "Materials and Methods."

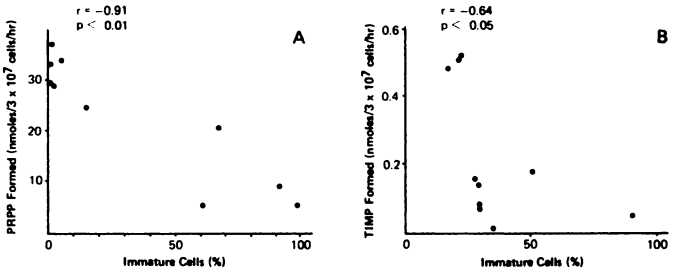

Chart 4. *A*, the PRPP formation in human leukemia leukocytes and immature cells. The same results as those presented in Chart 2 were plotted. *B*, production of TIMP and immature cells. The same results as those presented in Chart 3 were plotted.

cells than in immature cells ($t = -0.64$; $p < 0.05$) (Chart 4*B*). None of the patients studied had been treated with 6-MP.

The proposed hypothesis (5) that competitive utilization of PRPP between TIMP synthesis and other nucleotide synthesis resulted in a reduction of TIMP formation in cells was examined; the results are shown in Table 1. The effect of glutamine, a precursor of *de novo* synthesis, on the reaction catalyzing 6-MP and PRPP to TIMP was determined in a L1210 cell system in which the amount of PRPP was limited. Glutamine markedly inhibited the TIMP formation. Preincubation with glutamine increased the inhibitory effects, presumably by increased utilization of PRPP to form ribosylamine 5-phosphate. In comparison to L1210 cells, no PRPPATase activity was detectable in leukocytes from the patient with acute myeloblastic leukemia (Chart 5), which is in agreement with reported results (16). PRPPATase activity was found to be present in all tissues assayed with the exception of human leukemic leukocytes (Table 2). These results agree fairly with those reported by other workers (10, 15).

As shown in Table 3, inosine phosphorylase activity in homogenates of L1210 cells was much higher than that of other enzymes responsible for purine metabolism examined in this paper. Inhibition of inosine phosphorylase of homogenates of L1210 cells by 6-MP and thioinosine is also summarized in Table 3. From a plot of the inhibition rate on semilog paper, 50% inhibition was obtained with 1 × 10^{-3} M 6-MP. When thioinosine was used in place of 6-MP, the

Table 1

Effect of glutamine on the conversion of 6-MP to TIMP

The preincubation mixture contained the following components: 55 μmoles Tris buffer, pH 7.4; 5 μmoles $MgCl_2$; and 10,000 × *g* supernatant of L1210 cells; and was supplemented as indicated. [8-^{14}C]-6-MP was added after 10 min, and conversion to TIMP was measured by high-voltage electrophoresis.

Preincubation (10 min, 37°)		Incubation (10 min, 37°)		
Glutamine (μmoles)	PRPP (μmoles)	[^{14}C]-6-MP (μmoles)	PRPP (μmoles)	6-MP conversion to TIMP (%)
		0.05	0.1	82
	0.1	0.05		79
10		0.05	0.1	40
10	0.1	0.05		15

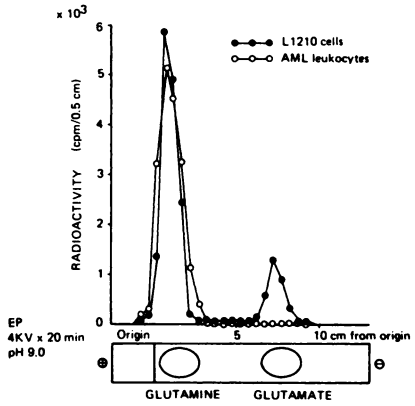

Chart 5. Chromatographic localization of ^{14}C following incubation of 10,000 × *g* supernatant of L1210 cells or acute myeloblastic leukemia (*AML*)leukocytes with L-[*U*-^{14}C]glutamine. L-[*U*-^{14}C]glutamine (100 nmoles) was incubated for 20 min with 10,000 × *g* supernatant of L1210 cells or of leukocytes from a patient with AML as described in "Materials and Methods." Aliquots of the reaction mixture were then developed by high-voltage electrophoresis.

Table 2

Phosphoribosylpyrophosphate amidotransferase activity in various tissues

The reaction conditions were the same as those described in "Materials and Methods."

Tissues	PRPPATase[a] activity
L1210 cells	27
L1210-bearing C57BL × DBA/2 F_1 mouse[b]	
Liver	59
Spleen	133
Human leukemic leukocytes	
SH 28F AML	<1
IT 50M CML	<1
MH 35F CML	<1

[a] nmoles of L-[*U*-^{14}C]glutamine per mg protein per hr.
[b] On the 7th day after inoculation.

result was almost the same. In order to clarify the metabolism of 6-MP itself, the above experiments were repeated with [8-^{14}C]-6-MP and inosine in place of 6-MP and [8-^{14}C]inosine. Furthermore, ribose 1-phosphate was used instead of inosine. The author could not show conversion of 6-MP to thioinosine in the presence of inosine or ribose 1-phosphate under the experimental conditions.

Table 3

Effects of 6-MP and thioinosine on the conversion of inosine to HX by L1210 cell homogenates

The formation of [8-^{14}C]HX from [8-^{14}C]inosine in L1210 cell homogenates was measured. The reaction conditions were the same as those described in "Materials and Methods" except that 6-MP and thioinosine were added in the concentrations indicated.

Inhibitor	Concentration (mM)	HX formed (nmoles/3 × 10^7/hr)	Inhibition (%)
None (control)		87.3	
6MP	0.05	78.5	10
6MP	0.5	54.2	39
6MP	1	46.6	47
Thioinosine	0.05	81.2	8
Thioinosine	0.5	76.7	12
Thioinosine	5	26.1	70

DISCUSSION

Results of our experiments have shown that the PRPP content of leukocytes is far less than the amount required for the formation of TIMP in leukocyte suspensions. Furthermore, TIMP formation in leukocyte homogenates was approximately 3 orders of magnitude higher than in cell suspensions, and the K_m value for PRPP in the reaction catalyzed by HGPRTase was 2 orders of magnitude higher than the PRPP contents (8, 9). Therefore, it seems likely that PRPP can be synthesized for TIMP formation in leukocyte suspensions during incubation with 6-MP. Since there were wide variations of TIMP formation among the cases examined, one would expect variation of PRPP formation in the cells. We did observe wide variation in the formation of PRPP in the cells. The cells in which the PRPP formation was relatively higher appeared to produce more TIMP. Thus, the availability of PRPP may play a key role in TIMP formation in human leukemic leukocytes. Recently, Scholar *et al.* (16) found that the lag in the initiation of TIMP formation in Sarcoma 180 cells was shortened by 6-methylmercaptopurine riboside and that the rate of TIMP formation was greater in the presence of 6-methylmercaptopurine ribonucleoside. It was suggested that the stimulation of 6-MP anabolism may be a consequence of an increased availability of PRPP.

In some mammalian tissues, PRPPATase is thought to play a major role in regulating *de novo* purine biosynthesis. Recent evidence has suggested that the intracellular levels of PRPP may be critical in controlling *de novo* and salvage purine biosynthesis. Since the concentration of PRPP is 1.3 × 10^{-4} M in L1210 cells, and the K_m for PRPP in PRPPATase and in HGPRTase are known to be 1.1 × 10^{-4} and 2.0 × 10^{-4} M, respectively, our results presented in Table 1 suggest that any changes in the activity of PRPPATase could effect corresponding changes in the TIMP formation in L1210 cells. The enzymes catalyzing the 1st step of *de novo* purine biosynthesis have been assayed by indirect methods. Recently, PRPPATase has been assayed directly in extracts prepared from lymphoblasts, placenta, hepatoma tissue culture cells, and spleen (20). However, there is no information regarding the direct assay of PRPPATase in human leukocytes. Although we attempted to determine whether

human leukemic leukocytes contained PRPPATase, the efforts were unsuccessful, in agreement with the results of other workers (17).

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