Comparative Study of 6-Mercaptopurine Metabolism in Human Leukemic Leukocytes and L1210 Cells

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Leukocytes from patients with leukemia and L1210 cells from mice were examined for the rate of formation and cellular concentration of phosphoribosylpyrophosphate, the rate of thioinosinic acid formation, and a number of selected enzymes involved in purine nucleotide synthesis. The amount of thioinosinic acid formed in L1210 cells was much higher than that in human leukemic leukocytes. In cell extracts, the synthesis of thioinosinic acid was similar in both cell types, and the amount of purine phosphoribosyltransferase was not rate limiting in either case. Much higher concentrations and rates of formation of phosphoribosylpyrophosphate were found in L1210 cells than in human leukemic leukocytes. The difference in response to 6-mercaptopurine between L1210 cells and human leukemic leukocytes might be attributed to their difference in supply of phosphoribosylpyrophosphate. Phosphoribosylpyrophosphate-amido-transferase was found to be high in L1210 cells, but was not detected in human leukemic leukocytes.

The enzymes responsible for purine nucleotide synthesis have been only partially studied in L1210 cells (4, 26). Few studies have been done on the characterization of the enzymes that are essential to the mechanism of action of 6-mercaptopurine (6-MP) in humans (5, 19, 20). It is well known that nucleotides can be formed by means of a *de novo* pathway from single metabolites or, alternatively, synthesized from preformed purine bases by means of salvage pathways. Synthesis of purine nucleotides from preformed purines in cells has been shown to be controlled more extensively by the endogenous supply of phosphoribosylpyrophosphate (PRPP) than by the activity of the purine phosphoribosyltransferase in experimental animal tumor cells (7), human erythrocytes (24), and lymphoblasts (6). To study the regulation of purine nucleotide synthesis and to clarify the mechanism of action of 6-MP in humans, we have undertaken comparative studies on the salvage and *de novo* pathways, the cellular concentration of PRPP, and the enzymes responsible for PRPP formation in human leukemic leukocytes and L1210 cells.

MATERIALS AND METHODS

Isolation of erythrocytes. Blood was obtained from patients with leukemia, none of whom had

been given 6-MP during the preceding month. Blood was drawn by venipuncture into heparinized tubes. The plasma and buffy coat were separated by centrifugation in the cold and then 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 (23). The cells were mixed with 1 volume of 3% dextran in 0.9% NaCl solution and allowed to sediment at room temperature for 30 to 45 min until a good separation was noted. The supernatant containing leukocytes, platelets, and some erythrocytes was decanted into a test tube in which all subsequent steps were performed. The cells were sedimented at $500 \times g$ for 10 min, washed with 0.9% NaCl solution, and sedimented at $500 \times g$ for 10 min. The cells were suspended in 0.9% NaCl solution and sedimented as described above. The leukocyte pellet was suspended in 1 ml of 0.9% NaCl solution. Contaminating erythrocytes were lysed by adding 3 volumes of water at 0°C. After 40 s, isotonicity was restored by adding 1 volume of 3.6% NaCl solution. The unbroken cells were collected by centrifugation at $500 \times g$ for 10 min, washed twice with 0.9% NaCl solution, resuspended, and centrifuged.

Isolation of L1210 cells. L1210 cells, harvested from mice 7 days after interperitoneal 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 thioinosinic acid (TIMP) formation. The leukocytes or L1210 cells were suspended in Eagle minimum essential medium containing 36% calf serum in a concentration of 3×10^7 cells per ml. The cell suspension (final volume, 10

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ml) was incubated at 37°C for 60 min in the presence of 0.05 μ mol of 6-[8-¹⁴C]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 Eagle minimum essential medium. The acid-soluble fraction was obtained by adding 0.05 ml of 10 M perchloric acid and then centrifuging. The residue was washed again with 1 ml of 0.5 M perchloric acid. The extracts were combined and neutralized with potassium hydroxide. After removal of insoluble perchloric acid, the neutralized extracts were chromatographed on a column of Dowex-1 formate resin (1 by 20 cm) with the appropriate carriers, with a formic acid gradient elution system (0 to 20 N). Column eluates were collected in 5-ml fractions with an automatic fraction collector. Nucleotides formed were determined by measuring the radioactivity of 0.5 ml of each fraction in toluene-based scintillation fluid by a Packard Tri-Carb scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). They are presented as nanomoles formed per 3×10^7 cells per h. Compared with the radioactivity of nucleotides in both human leukemic leukocytes and L1210 cells, little radioactivity was detected in the nucleoside region.

Extraction of PRPP. The sedimented leukocytes or L1210 cells, prepared as described above, were suspended in 1 ml of ethylenediaminetetraacetic acid (EDTA), pH 7.4, at a concentration of 2×10^7 cells per ml. The suspensions were deproteinized by heating in boiling water for 2 min and then promptly chilling in an ice bath. The denatured protein was sedimented by centrifugation, and the clear, supernatant fluid was used immediately for assaying the PRPP content. The recovery test showed that EDTA substantially prevented decomposition of PRPP (<10%) during the 2-min heating.

Assay of PRPP. The PRPP content of leukocytes and L1210 cells was determined by a modification of the procedure of Henderson and Khoo (8). The reaction mixture contained: 55 μ mol of tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4), 5 μ mol of MgCl_2 , 10 nmol of [8-¹⁴C]hypoxanthine, 0.09 ml of cell extract, and 0.1 ml of hemolysate (see below) in a final volume of 0.25 ml. After 20 min of incubation at 37°C, the reaction was stopped by adding 2 μ mol of neutralized EDTA, and a 20- μ l portion of the reaction mixture was spotted on Toyo no. 51 paper with appropriate carriers. Inosinic acid that was produced was separated from the substrate by high-voltage electrophoresis by using 0.05 M borate buffer (pH 9.0) containing 0.001 M EDTA at 4,000 V for 20 min (R_f values were: hypoxanthine, 0.03; inosinic acid, 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 amount of inosinic acid produced. The standard curves showed a linear responsiveness of up to 5 nmol of PRPP. Little radioactivity was detected in the nucleoside region under the conditions used.

Enzyme preparations. Erythrocytes were hemolyzed by adding 1 ml of 0.2 M Tris buffer (pH 7.4) to 2×10^8 packed cells, and the hemolysates were used

as the crude hypoxanthine guanine phosphoribosyltransferase (HGPRTase) for assay of PRPP. Leukocytes or L1210 cells (3×10^7) were suspended in 0.1 ml of 0.001 M Tris buffer (pH 7.4) and were homogenized with a Teflon homogenizer for 40 s. The homogenates were used to determine purine phosphoribosyltransferase. Leukocytes or L1210 cells were also suspended in 1 volume of 0.2 M Tris buffer (pH 7.4) and homogenized as described above. After centrifugation at $10,000 \times g$ for 10 min, the supernatant fluid was used for the assay of phosphoribosylpyrophosphate-amidotransferase (PRPPATase).

Enzyme assays. Purine phosphoribosyltransferase activity was assayed by the method of Kelley et al. (13). The final reaction mixture (100 μ l) contained: 5.5 μ mol of Tris buffer (pH 7.4), 0.5 μ mol of MgCl_2 , 0.1 μ mol of PRPP, 0.05 μ mol of 6-[8-¹⁴C]MP or 0.05 μ mol of [8-¹⁴C]hypoxanthine, and homogenates of 2×10^6 leukocytes. After incubation for 10 min at 37°C, the reaction was terminated by adding 2 μ mol of neutralized EDTA. A 20- μ l portion of the reaction mixture was spotted on Toyo no. 51 paper with appropriate carriers. Nucleotides produced were separated from the substrate, and the radioactivities of the substrate and the separated nucleotides were determined under the same electrophoretic conditions as those described in the assay of PRPP (R_f values were: 6-MP, 0.45; TIMP, 1.0). Nucleotide formation showed a linear relationship between incubation time and cell number. PRPPATase was assayed by the radiochemical method of Tay et al. (25). The reaction mixture contained: 55 μ mol of Tris buffer (pH 7.4), 5 μ mol of MgCl_2 , 100 nmol of L-[U-¹⁴C]glutamine, 30 nmol of PRPP, 2.5 μ mol of dithiothreitol, and 1 to 2 mg of protein in a final volume of 0.25 ml. After 20 min of incubation at 37°C, the reaction was stopped by adding 2 μ mol of neutralized EDTA. A 20- μ l portion 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, 0.02; 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 between incubation time and protein concentration. Protein was measured by the method of Lowry et al. (15).

RESULTS

It has been shown that 6-MP must be converted to TIMP to have a biologically active effect (3). The amounts of TIMP formed in leukocytes from leukemic patients (two with acute leukemia and eight with chronic leukemia) were determined, which ranged from 0.01 to 0.52 nmol/ 3×10^7 cells per h (Table 1). The

TABLE 1. Parameters of purine nucleotide metabolism and TIMP formation in L1210 cells and leukemic leukocytes

Determination	TIMP formed (nmol/3 × 10 ⁷ per h)	6-MP (nmol/3 × 10 ⁷ per h)	Hypoxanthine (nmol/3 × 10 ⁷ per h)	PRPP concn (nmol/3 × 10 ⁷)	PRPP formed ^c (nmol/3 × 10 ⁷ per h)	PRPPATase (nmol of glutamate/mg of protein per h)
Leukemic leukocytes	0.01 to 0.52 (10) ^a	329 ± 56 ^c (7)	765 ± 77 (7)	<0.03 (3)	5.4 to 38.0 (10)	<1 (3)
L1210 cells	1.95 ± 0.25 ^a (3)	425 ± 99 ^c (3)	915 ± 150 ^c (3)	4.9 ± 0.9 ^d (3)	830.4 ± 75.2 ^d (3)	27 ± 5 ^d (3)
L1210-bearing BDF ₁ mouse/ ^e Liver Spleen						59 ± 8 (3) 133 ± 15 (3)

^a Cells (10⁷) were suspended in 1 ml of 0.9% NaCl solution containing 10 mM glucose and 60 mM inorganic orthophosphate. The suspensions were incubated for 60 min at 37°C.

^b Numbers in parentheses are number of subjects studied.

^c Mean ± standard deviation.

^d $P < 0.01$.

^e $P > 0.05$.

^f On day 7 after inoculation.

amounts of TIMP formed in L1210 cells, 1.95 ± 0.25 nmol/3 × 10⁷ cells per h, were much higher than those in human leukemic leukocytes. This synthetic reaction is catalyzed by purine phosphoribosyltransferase and requires PRPP for the substrate (3). There were no significant differences in HGPRTase and 6-MP phosphoribosyltransferase activity between L1210 cells and human leukemic leukocytes. (Table 1). Furthermore, purine phosphoribosyltransferase in leukemic leukocytes did not show wide variations in activity.

The PRPP content in heat extracts of leukocytes from the patients with acute leukemia and of L1210 cells is also given in Table 1. By using our radiochemical assay, it was possible to detect amounts of PRPP as low as 0.03 nmol/3 × 10⁷ cells in leukocytes. However, no significant radioactivity was detected in the nucleoside region in any of the leukocytes by the electropherograms. Our results indicate that such amounts, if present in leukocytes, are less than the amounts of TIMP formed in leukocyte suspensions. In contrast, considerable amounts of PRPP (4.9 ± 0.9 nmol/3 × 10⁷ cells or 0.16 ± 0.4 μmol/g) were found in L1210 cells. This value agrees fairly well with the results reported by Henderson and Khoo (0.28 μmol/g in Ehrlich ascites carcinoma cells) (8) and Paterson and Wang (0.18 μmol/g in Ehrlich ascites carcinoma cells) (17).

Also, synthesis of PRPP during incubation of leukemic leukocytes was determined. Even after incubating leukocytes in Eagle minimum essential medium at 37°C for 60 min, the intracellular concentrations of PRPP did not increase to the threshold of the assay procedure. Since Hershko et al. (9) reported that PRPP formation was enhanced by inorganic orthophosphate, we examined its effects on PRPP formation in leukocyte suspensions. After 1 h of incubation in the presence of 60 mM inorganic orthophosphate, there was a marked elevation of the PRPP concentration. The amount of PRPP formed in leukocytes from 10 patients with acute leukemia ranged from 5.2 to 38.0 nmol/3 × 10⁷ cells per h. In contrast, the amount of PRPP formed in L1210 cells was much higher (Table 1).

It has been demonstrated by Wood and Seegmiller (27) and Holmes et al. (11) that PRPPATase, the enzyme catalyzing the first step of de novo purine synthesis, exhibits allosteric properties and is subject to feedback regulation by purine nucleotide end products. Because of the apparent regulatory importance of this enzyme in de novo purine synthesis, we determined whether leukemic leukocytes or L1210 cells contained it by directly measuring the rate of

PRPP-dependent glutamate production from glutamine. In leukocytes from patients with acute leukemia, little radioactivity was detected in the glutamate region compared with that in L1210 cells. These results agree with those of others who have shown that leukocyte suspensions lack the initial reaction for de novo purine synthesis (21, 22) and that PRPPATase activity is present in all tissues assayed except human leukemic leukocytes. These values agree fairly well with the figures presented by other workers (10, 18) (Table 1).

DISCUSSION

Though the varied response of human leukemic leukocytes to 6-MP is well known, L1210 cells are much more sensitive to this agent (1, 2). It is interesting to consider whether these differences in response represent biochemical differences in 6-MP anabolism. Since many workers have reported that the differences in sensitivity of human leukemic leukocytes and L1210 cells may be related to their capacity to synthesize TIMP (14), one would expect differences in their capacity to convert 6-MP to TIMP. As shown here, TIMP formation was greater in L1210 cells than in leukemic leukocytes. It has been shown recently that the rate of purine nucleotide salvage synthesis in cells is limited by the supply of PRPP, the activity of purine nucleotide pyrophosphorylase, and purine base transport into cells (6).

The activities of purine phosphoribosyltransferase in leukemic leukocytes and L1210 cells studied here were reasonably similar and were not rate limiting. L1210 cells, however, had a considerable amount of PRPP, compared to leukemic leukocytes. Furthermore, the amount of TIMP formed in L1210 cells was also much higher than that formed in leukemic leukocytes. Therefore, it seems probable that the differences in capacity of the cells to convert 6-MP to TIMP may be due to its supply of PRPP. The amount of PRPP in leukemic leukocytes is far below the K_m value for PRPP in purine nucleotide synthesis (20) (Table 1). It is, therefore, likely that the availability of PRPP may be an important factor in regulation of purine nucleotide synthesis. Although there are some reports on the supply of PRPP in erythrocytes, there is no information regarding the supply of PRPP in human leukemic leukocytes and L1210 cells. The synthesis of PRPP from adenosine 5'-triphosphate and ribose 5-phosphate is considered to be regulated by the availability of ribose 5-phosphate (12). It has also been shown that glucose 6-phosphate dehydrogenase activity is the key enzyme in hexose monophosphate shunt functioning, through which ribose 5-

phosphate is channeled (12). It is interesting to consider whether the differences in supply of PRPP found here represent biochemical differences in hexose monophosphate shunt in the cell types.

There has been no reported information regarding the direct assay of PRPPATase in human leukemic leukocytes. The present study showed that PRPPATase activity is found in L1210 cells, but not in human leukemic leukocytes, which agrees with the results of other workers obtained by indirect assay (21, 22). The difference in PRPPATase activity between the two cell lines is interesting, for it enables us to discuss the antileukemic effect of another antimetabolite, methylthioinosine. This compound is able to inhibit growth of some tumor cells, which might be due to the inhibition of PRPPATase by the methylthioinosine nucleotide. In fact, Luce et al. (16) concluded that methylthioinosine was ineffective as an antileukemic agent in patients with acute leukemia, although this agent was very effective in the treatment of mouse L1210 cell leukemia. The difference in effectiveness might be explained on the basis of PRPPATase activity in the two cell lines.

In nonhuman systems, the most frequent cause of acquired insensitivity to 6-MP has been the loss, or marked decrease, of HGPRTase activity (3). In contrast, to date, a severe deficiency of HGPRTase in 6-MP-resistant human leukemic leukocytes has been reported by Davidson and Winter (5) in only 1 of 14 patients, by Rosman and Williams (20) in only 1 of 8 patients, and by Rosman et al. (19) in only 1 of 18 patients. These results indicate that this mechanism might not be operative in most human leukemia (5, 19, 20). The relative low frequency of severe deficiency in HGPRTase in human leukemia compared with murine neoplasms and bacterial systems may be a consequence of little or no de novo biosynthesis of purine nucleotides by the leukemic leukocytes (21).

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