The Metabolism of Thioinosinic Acid by 6-Mercaptopurine Sensitive and Resistant Leukemic Leukocytes[1] (36472)

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The conversion of 6-mercaptopurine (MP) to the 5′-nucleotide must occur before MP is an active antimetabolite. This critical reaction is catalyzed by hypoxanthine–guanine phosphoribosyltransferase (HGPRTase) through a reaction with 5-phosphoribosyl-1-pyrophosphate (PRPP). The role of this transformation in the susceptibility and resistance of L1210 leukemia and P388 murine lymphocytic leukemia to MP in culture has been studied by Brockman (1) and Davidson (2) who found that resistance developed in both cell lines when selected cells failed to metabolize the purine analogue to the 5′nucleotide, thioinosinic acid. The same mechanism of bacterial resistance to MP has also been demonstrated in *Streptococcus faecalis* and *Salmonella typhimurium* (3, 4). Human leukemia resistance to MP does not seem to be due to a failure of resistant cells to convert MP to thioinosinic acid. Davidson and Winter (5) investigated HGPRTase activity in leukocytes from patients with leukemia who had been treated with MP and subsequently developed resistance to the drug. In carefully controlled experiments they demonstrated that in only one case of the 15 clinically resistant patients tested was there a measurable decrease in leukocyte HGPRTase activity. We have also observed good HGPRTase activity in leukocytes from leukemia patients resistant to MP therapy.

The available evidence suggests that while a loss of HGPRTase can happen in man, it is uncommon and other mechanisms of MP resistance occur in human leukemia. This paper describes the effects of extracts from both MP resistant and sensitive leukemic leukocytes on ^{14}C-thioinosinic acid and the identification of the resulting products.

Methods. Preparation of labeled thioinosinate. ^{14}C-Thioinosinate was synthesized enzymatically by reacting 8-^{14}C-MP with PRPP in the presence of a six-fold purified *Escherichia coli* HGPRTase preparation according to conditions described by Carter (6). Unreacted MP and thioinosinic acid were separated by elution from a Reyxon 201 (200–400 mesh, Cl^-) column with 0.05 *M* HCl to remove MP followed by elution with 0.5 *M* HCl to remove thioinosinic acid. Thioinosinate and unreacted MP were detected in the eluate by reading fractions at 320 nm in 3-ml quartz curvettes (1-cm light path) with a Beckman DU spectrophotometer. Fractions containing thioinosinic acid were combined and adjusted to pH 9 with 10 *M* NaOH.

The purity of the enzymatic product was verified chromatographically by developing samples on Whatman No. 1 chromatography paper with a Na_2HPO_4 (5%)–isoamyl alcohol (1:1) solvent. Spots were detected with UV light using a model C-3 Chromato-view cabinet (Ultraviolet Products, Inc.). Radioactivity was confined exclusively to the UV-fluorescent area having an R_f identical to authentic thioinosinate controls developed on the same chromatogram. Synthetic thioinosinate was obtained through a generous gift from the Southern Research Foundation. Radioactivity was measured with a Packard

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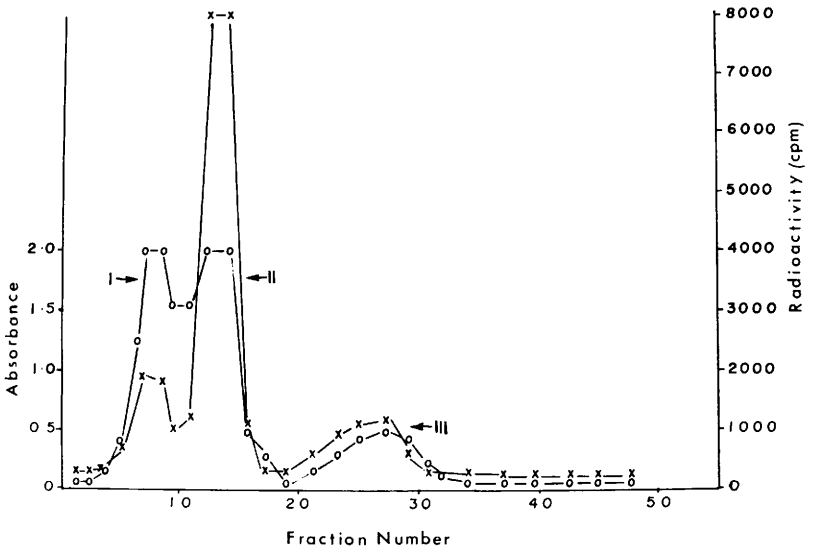

FIG. 1. Distribution of 250 nm absorbing substances and radioactivity from the reaction of leukocyte extracts on ^{14}C-thioinosinic acid. O—O Absorbance (250 nm); X—X Radioactivity (cpm).

Tri-Carb scintillation counter. Appropriate corrections for background and quenching were made for all determinations.

Separation of leukocytes from whole blood. Leukocytes from peripheral blood of patients with acute lymphocytic leukemia (All) and from normal donors were prepared by dextron sedimentation followed by a 30-sec hypotonic shock to remove residual red cells (7). Leukocytes were then frozen and stored at —40° until used.

Preparation of leukocyte extracts. Frozen leukocytes were thawed, suspended in 0.05 *M* phosphate buffer and ruptured with a MSE sonic oscillator. Three 30-sec bursts at maximum output with 2-min cooling intervals were used on all cell suspensions. Samples were centrifuged at 10,000*g* for 15 min and the supernatant further centrifuged at 100,000*g* for 1 hr. Extracts were dialyzed for 1 hr against 500 ml 0.05 *M* phosphate buffer (pH 7.2). Protein concentrations were determined by the Lowry method using crystalline bovine serum albumin as a standard (8). All procedures were carried out at 5° except where indicated in the text.

Leukocyte extracts were reacted with thioinosinate under the following conditions: A mixture containing 8-^{14}C-thioinosinate (2.4 μM) and extract in 30 ml 0.05 *M* phosphate buffer (pH 7.2) was incubated for 2 hr at 25°. The reaction flask was then placed in a boiling water bath for 3 min, immediately cooled in an ice bath and the solution centrifuged at 10,000*g* for 10 min. The supernatant was concentrated to approximately 5 ml *in vacuo* and 2 mg each of unlabeled inosine monophosphate (IMP), thioinosinate and MP were added to the concentrate. The mixture was applied to a 400-mm Belco column packed to a height of 25 cm with Rexon 201 (Cl^-) ion-exchange resin and reaction products eluted from the column with 0.01 *M* HCl. Optical densities were determined on each fraction at 250 and 320 nm with a Beckman DU spectrophotometer and the radioactivity estimated by counting a measured sample of each fraction.

Results. When labeled thioinosinate was incubated with leukocyte extracts under the conditions described, three to four radioactive products were usually detected. An elution profile from one of the experiments (P-198) is shown in Fig. 1. The distribution of radioactivity followed that of two 250 nm absorbing peaks while a third substance was

TABLE I. Reaction of Acute Lymphocytic Leukemia Extracts on ^{14}C-Thioinosinate.

Patient	Sex	MP status when studied	Thioinosinate[a] converted to IMP and hypoxanthine μmole/mg protein	Thioinosinate[a] converted to MP μmole/mg protein
326	M	Resistant	0.05	0.03
326[b]	M	Resistant	0.12	0.002
487	F	Resistant	0.02	0.01
487[b]	F	Resistant	0.06	0.01
422	M	Resistant	0.02	0.01
P-198	M	Resistant	0.10	None detected
P-40	M	No response to any treatment	0.01	None detected
P-79	M	New patient	0.05	None detected
P-51	M	New patient	0.04	0.01
367	F	Sensitive	0.007	None detected
P-103	M	Sensitive	0.002	0.04

[a] $\frac{\text{Total counts in products}}{\text{Total initial counts in } ^{14}\text{C-thioinosinate}} \times 100 = \%$ product formed from substrate;

$\frac{\%\text{ product formed}}{\text{Total mg protein in reaction mixture}} \times 2.4 = \mu$moles product formed/mg protein

[b] Samples taken 5 days after first sample. (Neither patient responded to further therapy.)

eluted with a radioactivity distribution close to but not identical to that of a substance also absorbing UV at 250 nm. A fourth product was observed in some experiments. This material had a radioactivity distribution identical to the UV absorbance of free MP (320 nm).

Fractions from each peak showing the highest count were concentrated and further characterized chromatographically. Each substance was developed overnight with an isoamyl alcohol–5% Na_2HPO_4 (1:1) solvent on Whatman No. 1 chromatography paper. Authentic samples of hypoxanthine, inosine and IMP were developed on the same chromatogram as controls. Spots were visualized under UV illumination, and radioactivity estimated by counting small sections of the paper covering the entire length from the origin to the solvent front. The first substance (peak I) had an R_f identical to that of authentic IMP and the second substance was identical to the R_f of authentic hypoxanthine. The distribution of radioactivity was confined to the area of UV absorbance in each case. In samples where labeled MP was detected, the product was characterized as free MP by the same criteria. Samples from peaks I and II partially characterized as IMP and hypoxanthine, were reacted with 5′-nucleotidase (Sigma Chemical Co.) and xanthine oxidase (Worthington Biochemical Corp.), respectively. The reaction mixtures were developed on Whatman No. 1 chromatography paper overnight with an isoamyl alcohol–Na_2HPO_4 solvent, and the distribution of radioactivity determined for both reactions. The product of the reaction of peak I with 5′-nucleotidase had an R_f identical to that of authentic inosine and was labeled. In a like manner, the product of the reaction of peak II and xanthine oxidase was labeled and had an R_f identical to that of authentic uric acid. The reaction products absorbing UV at 250 nm were identified as IMP and hypoxanthine, respectively. The third 250 nm absorbing substance shown in Fig. 1 is as yet unidentified.

Results of the effects of leukocyte extracts from both MP resistant and sensitive leukemia patients are tabulated in Table I, and data obtained with extracts from normal leukocytes are recorded in Table II. Values for

TABLE II. Reaction of Normal Leukocyte Extracts on ^{14}C-Thioinosinate.

Source of cells	Thioinosinate converted[a] to IMP and hypoxanthine μmole/mg protein	Thioinosinate[a] converted to MP μmole/mg protein
Blood bank (1)	0.006	0.0005
Blood bank (2)	0.007	0.0005
Lymphocytes (Thymus)	0.012	0.0005

[a] See Table I for calculations.

thioinosinate conversions to 250 nm absorbing substances (IMP and hypoxanthine) by the various extracts were calculated from the total radioactivity contained in both fractions. Our reasoning for this is (i) the extracts are inactive on free MP and (ii) leukocyte extracts rapidly convert the riboside of MP back to the free base and ribose. The data indicate that thioinosinate was converted first to IMP and the presence of hypoxanthine in column eluates was due to further enzyme activity on IMP. Under the conditions employed, leukocyte extracts convert thioinosinate to IMP and in the majority of samples there is a low but measurable conversion of the drug back to MP. Specific activities of extracts from MP resistant leukocytes appear to be greater for the conversion of the labeled drug to IMP than the values obtained with MP sensitive leukemia cells or with normal leukocytes.

Discussion. The conversion of MP to normal purine has been reported in growing *Bacillus cereus* cells (9). Coggin, Loosemore, and Martin (10) also observed the metabolism of MP to normal purines in *Escherichia coli,* and further showed with crude extracts that this conversion likely occurred after thioinosinate was first transformed to IMP. The observed conversion of thioinosinate to IMP by leukocyte extracts may explain in some instances the development of clinical resistance to MP. Enzymatic alterations of thioinosinate would tend to lower intracellular concentrations, and secondly, hypoxanthine, presumably as IMP, is known to reverse the cytotoxic effects of MP (11). Hence, the observed transformation of thioinosinate to IMP would have two effects; that of altering the drug concentration within the cell and that of forming a metabolite which can overcome inhibitory effects of the drug.

While the data suggest that there is an increased conversion of labeled thioinosinate to IMP in resistant cells, they must be interpreted with caution. Transformations observed in leukocyte extracts from three patients (367, P-40 and P-103) were of the same order of magnitude as those seen in normal leukocytes while conversions observed with cells from patients P-79 and P-51 were higher. The subsequent response to MP in two patients is not known since they both died of complications before its effect could be determined. A third patient, P-51, had a remission lasting three months while receiving Prednisone with Vincristine and cytosine arabinoside with MP; this response may or may not have been due to MP sensitivity. The two lowest values obtained at diagnosis (367 and P-103) were in patients who did have a therapeutic response to MP.

Resistant cells were obtained from patients in relapse. These patients had all received a therapeutic response to MP earlier in the course of their disease, but did not respond to further MP therapy. Conversion rates observed in extracts from these cells were significantly higher than those observed in normal leukocyte extracts or with MP sensitive leukemic leukocytes. The second blood samples from patients 236 and 487 were obtained in both cases just before the patients expired, and the observed increase in conversion rates is likely due to the advanced stages of the

disease rather than to further MP therapy. In all other resistant cases, values were obtained with single blood samples. The condition of patients in relapse usually did not permit our taking multiple blood samples and the quantity of blood taken was such that the leukocytes recovered were sufficient for one determination only. Hence, the specific activities recorded for the observed conversions must be interpreted as suggestive rather than significant in terms of absolute values.

Of additional interest, is the observation that some leukocyte extracts metabolize a portion of thioinosinate back to the free base (MP). The degree of conversion varies considerably with no apparent relationship to resistance. This reaction does not appear to be an artifact. Mixtures identical to those used in experiments were incubated without enzyme and separated by the same procedure. No significant levels of radioactivity were observed in any of the fractions except that of the original material. Secondly, there was always a relationship between protein present in the reaction mixture and conversions observed (*i.e.*, the more protein the greater the conversion). The formation of free MP from thioinosinate is likely the product of two separate enzymes; a phosphatase followed by a nucleoside phosphorylase. We have observed the latter reaction a number of times in leukocyte extracts, and have some evidence for the presence of phosphatase active on thioinosinate. It may be that the conditions we employed with extracts are not optimum for the full catalytic activity of both enzymes while they may play a more significant role in MP resistance in the intact cell.

MP is a purine and as such closely adheres to the known pathways for the anabolism and catabolism of purine bases. The analog is converted to thiouric acid by xanthine oxidase (9). It has been shown to be affixed to deoxynucleotides producing MP deoxyribotides (12). In addition to the well known conversion of MP to thioinosinate by HGPRTase, the riboside of MP is rapidly cleaved to the base by Ehrlich ascites tumor cells (13), and by the leukocyte extracts reported here. Hence the capability of degrading MP by pathways general to purine catabolism is implied if not already documented in the considerable literature on the drug.

Leukocytes obtained from normal blood consist of a mixture of granulocytes, monocytes and lymphocytes and are not strictly comparable to the lymphoblastic leukemias studied. What is important is that the capacity to convert thioinosinic acid to IMP is a property common to both normal and neoplastic leukocytes, and in most cases tested, both cell types have the ability to catabolize thioinosinate back to its free base. The development of clinical resistance to MP in some cases may be due to the selection of a cell population with an increased capacity (i) to convert thioinosinate to normal mebolites, (ii) to degrade the drug back to its free base or (iii) a combination of both. It is possible that such cell populations would still be sensitive to higher drug concentrations, but because of the general toxicity of this drug on myelopoiesis, the systemic levels of MP which can be tolerated are severely limited. Under such circumstances, the conditions for the selection of a resistant cell population are not as restricted as they are when the concentration of MP may be varied over a much wider range, and may explain the difference in the mechanism of MP resistance observed in tissue culture versus that which may occur in patients.

Summary. The antileukemic drug, 6-mercaptopurine is cytotoxic only after it is converted to the 5′-nucleotide, thioinosinic acid. When ^{14}C-thioinosinic acid was incubated with extracts from both MP sensitive and MP resistant leukocytes a portion of the drug was converted to inosine and hypoxanthine. The conversion of thioinosinate back to the free base was also observed in some preparations, but not all. The conversion of thioinosinic acid to IMP and hypoxanthine appeared to be greater in extracts from drug resistant leukocytes than in extracts from sensitive cells. There was no apparent relationship between resistance and the conversion of thioinosinic acid back to free 6-mercaptopurine.

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