Biochemical Parameters of Mercaptopurine Activity in Patients with Acute Lymphoblastic Leukemia

Solomon Zimm,1 Gregory Reaman, Robert F. Murphy, and David G. Poplack2

Pediatric Branch, National Cancer Institute, NIH, Bethesda, Maryland 20205 [S. Z., R. F. M., D. G. P.], and Children's National Medical Center, Washington, DC [G. R.]

ABSTRACT

Mercaptopurine (MP) is a purine antimetabolite widely used for remission maintenance in the therapy of acute lymphoblastic leukemia. In order to study the biochemical parameters affecting MP activity, leukemic cells were obtained from ten patients with acute lymphoblastic leukemia at the time of diagnosis and from the same patients at the time of their initial marrow relapse. Hypoxanthine phosphoribosyltransferase (HPRT), the enzyme that converts MP to its active, nucleotide metabolite, thioinosine monophosphate; alkaline phosphatase, the primary catabolic enzyme of thioinosine monophosphate; and 5-phosphoribosyl-1-pyrophosphate (PRPP), the cellular ribose-phosphate donor essential for MP activation, were all measured within the patients' leukemic cells.

There was marked interpatient variability in the three biochemical parameters studied with a greater than 10-fold range in alkaline phosphatase activity and an approximately 100-fold range in HPRT activity and PRPP levels. Four patients developed changes in biochemical parameters that influence MP activity at the time of relapse. In three of the four patients, alterations in more than one of these three biochemical parameters were noted. Three of four patients had a greater than 50% decrease in intracellular HPRT activity, four of four had a greater than 50% decrease in intracellular PRPP, and two of four had a greater than 9-fold increase in intracellular alkaline phosphatase activity at relapse. Two of four patients demonstrated changes in all three parameters at relapse in the directions that could have resulted in decreased MP sensitivity (i.e., decreased HPRT, decreased PRPP, and increased alkaline phosphatase). There was no correlation between pretreatment values of HPRT, PRPP, and alkaline phosphatase and remission duration.

These results indicate that: (a) there is marked variation in HPRT, PRPP, and alkaline phosphatase in patients with acute lymphoblastic leukemia and b) following MP-containing maintenance chemotherapy, some patients develop biochemical changes that may result in decreased sensitivity to MP.

INTRODUCTION

Although greater than 90% of children with ALL3 achieve an initial complete remission with current therapy, nearly 50% of these patients will relapse during or following cessation of maintenance chemotherapy (1). In most maintenance regimens, chemotherapy consists primarily of daily, p.o. MP and weekly methotrexate. The relatively high relapse rate observed in this disease suggests that acquired resistance to MP may be an important clinical problem. In order for MP to be biologically active, it must undergo intracellular conversion to a nucleotide, TIMP (2). This compound is inhibitory to several enzymes in the de novo purine pathway (2), and it may also undergo further metabolism to thioguanine nucleotides which are then incorporated into DNA and RNA (3). Either or both of the above may be responsible for the drug's antineoplastic effect. HPRT is the enzyme that catalyzes the reaction in which MP is converted to TIMP, and PRPP is the essential cofactor that serves as the ribose-phosphate donor.

Acquired MP resistance in murine leukemias and solid tumors has been studied in vitro and found to result most frequently from a marked decrease in HPRT activity (4). Increased alkaline phosphatase activity, an enzyme which can degrade TIMP to mercaptopurine riboside, has also been reported as the cause of MP resistance in a Sarcoma 180 cell line (5).

However, despite its clinical use for over 30 yr, little is known regarding the clinical biochemical determinants of MP activity. The present study was conducted in order to examine HPRT and alkaline phosphatase activities and PRPP levels in patients with ALL at diagnosis and first relapse in order to determine: (a) if these factors are affected by chronic MP-containing maintenance chemotherapy; (b) if there is a large degree of interpatient variability in these biochemical parameters; and (c) if alterations could occur in these factors that might result in decreased sensitivity to MP.

MATERIALS AND METHODS

Patients. Leukemic cells were obtained from ten patients at the time of their diagnosis and at the time of their first bone marrow relapse. All patients had a histologically confirmed diagnosis of acute lymphoblastic leukemia. The patients were all treated on the same protocol (6) in which maintenance chemotherapy consisted of p.o. mercaptopurine, methotrexate, and cyclophosphamide along with cyclic, periodic reinduction therapy. Six patients were male, and four were female. All but one of the patients (who had T-cell ALL) were considered to be in a high-risk prognostic category according to the criteria of the Children's Cancer Study Group.

Leukemic Cells. The leukemic cells were obtained from peripheral blood samples of patients in whom greater than 80% of circulating WBCs were lymphoblasts. Heparinized blood samples were obtained on ice, and the mononuclear cell layer was separated by Ficoll-Hypaque gradient centrifugation (7). Cell viability exceeded 90% by trypan blue dye exclusion. The cells were then frozen in 10% dimethyl sulfoxide at −190°C until the time of analysis. The cells were then rapidly thawed and lysed, and the cell supernatants were prepared for the various enzyme and cofactor assays.

HPRT Assay. HPRT activity was measured by quantitation of the conversion of radiolabeled hypoxanthine to inosine monophosphate (8, 9). Cells were washed 3 times in phosphate-buffered saline, suspended...
in 1 ml of distilled water, and lysed by four cycles of freeze-thawing. The resulting lysate was centrifuged for 30 min at 20,000 × g. The supernatant was heated for 10 min at 60°C and then assayed. The assay incubate consisted of 5 mM MgCl₂, 1 mM PRPP, 0.1 mM glycine buffer, pH 10, 0.15 mM [¹⁴C]hypoxanthine (49 nCi/mmole), and 20 μl of cell supernatant in a total volume of 100 μl. The incubation was carried out for 15 min and terminated by placing the samples on ice and adding 1.25 mM of EDTA in 5 μl. Twenty-five-μl aliquots were then spotted on Whatman DE-81 filter disks. The samples were then allowed to dry and then washed: 3 times with 1 mM ammonium bicarbonate; once with distilled water; and then once with methanol. After drying, radioactivity bound to the disks, indicating the amount of nucleotide formed, was measured in a liquid scintillation counter.

**Alkaline Phosphatase Assay.** Cells were suspended in distilled water and, while being kept at 4°C, were sonically disrupted by three 15-s bursts using an Artek sonic dismembrator at a setting of 35. After the cell extracts were made up to a final concentration of 0.05 mM Tris-HCl, pH 7.6, they were dialyzed overnight against two 1-liter exchanges of 0.05 mM Tris-HCl, pH 7.6. Alkaline phosphatase was determined at 37°C by measuring the rate of hydrolysis of p-nitrophenylphosphate. This was done by following the change in absorbance at 410 nm over 20 min. The reaction mixture contained 2.8 ml of 100 mM carbonate-bicarbonate buffer, pH 9.2, 11 mM p-nitrophenylphosphate, and 11 mM MgCl₂, and the reaction was initiated by the addition of 0.2 ml of the cell sonicate (10).

**PRPP Assay.** The intracellular concentration of PRPP was determined by measuring the release of ¹⁴C0₂ from [carboxyl-¹⁴C]orotic acid in the presence of the combined enzyme, orotate phosphoribosyltransferase-decarboxylase, which catalyzes a PRPP-dependent reaction. Cells were lysed by suspending them in 10 mM Tris-1 mM EDTA, pH 7.4, and then immersing them in boiling water for 30 s. The lysates were then centrifuged at 10,000 × g for 15 min at 4°C. Following this, the supernatants were assayed for PRPP as previously reported (11).

**Protein.** Cell lysate protein concentrations were determined by the method of Lowry et al. (12).

**RESULTS**

The HPRT, alkaline phosphatase, and PRPP values measured in the ten patients at diagnosis and at the time of their first bone marrow relapse are shown in Table 1. There was marked interpatient variability noted in all three biochemical parameters with a greater than 10-fold range in alkaline phosphatase and an approximately 100-fold range in HPRT and PRPP. The mean values for HPRT, alkaline phosphatase, and PRPP did not differ significantly between diagnosis and relapse in the overall group of patients. In addition, there was no correlation between remission duration and the initial values for HPRT, PRPP, and alkaline phosphatase in the patients studied. However, when the patients’ HPRT, alkaline phosphatase, and PRPP values at diagnosis and relapse were examined individually, differences in the biochemical parameters studied were observed.

Three of the ten patients studied (Patients 1, 6, and 9) had a greater than 50% decrease in HPRT activity at relapse compared to their values at diagnosis.

A marked increase (9-fold) in alkaline phosphatase activity occurred in two patients (Patients 6 and 9) at the time of their first relapse.

In four patients (Patients 1, 4, 6, and 9), a greater than 50% decrease in PRPP levels was detected at the time of relapse, while one patient had a 50% rise in PRPP levels at relapse.

Of the ten patients studied, four developed biochemical changes that might have resulted in decreased sensitivity to MP at the time of first marrow relapse. Three of four patients developed changes in more than one parameter at relapse (Patients 1, 6, and 9). One patient developed a greater than 50% decrease in PRPP (Patient 4). In another patient, there occurred a 50% decrease in both HPRT and PRPP at relapse (Patient 1). Two patients (Patients 6 and 9) developed changes in all three biochemical parameters at relapse, that is, greater than 50% decreases in HPRT and PRPP as well as large increases in alkaline phosphatase activity.

Comparison of remission duration in these patients revealed that, in patients without biochemical changes, the mean remission duration was 18.3 mo, while in those with biochemical changes, the mean remission duration was 10.8 mo. These differences, however, were not statistically significant.

**DISCUSSION**

The substantial relapse rate that occurs in patients with ALL during and following completion of maintenance chemotherapy,
which includes daily p.o. MP, suggests that acquired MP resistance is a clinical problem. In contrast to the in vitro setting, where decreased HPRT activity is the most frequently described mechanism by which cells become resistant to MP, little is known regarding mechanisms by which clinical MP resistance arises. Three biochemical parameters that determine the extent of MP activation are the activities of the enzymes HPRT, alkaline phosphatase, and the concentration of PRPP. Although there is clear evidence for their importance in the in vitro setting, their influences on the clinical efficacy of MP have not been well documented. The present study was conducted in order to examine HPRT, alkaline phosphatase, and PRPP levels in patients with ALL treated with MP. In contrast to previous studies, all three factors were measured at the time of diagnosis and first bone marrow relapse with each patient serving as his own control. All patients had ALL and were treated on the same protocol.

In contrast to the in vitro setting, previous work has suggested that decreased HPRT did not appear to be a major means by which cells became MP resistant in the clinical setting (10, 13). In the current study, decreased HPRT was noted in three of ten patients. Although the degree of decrease was approximately 50%, it is not clear that a decrease in HPRT activity of this magnitude would result in MP resistance.

It has previously been suggested that increased alkaline phosphatase activity could contribute to the emergence of clinical MP resistance (14). Increased alkaline phosphatase was seen at relapse in two of the ten patients examined in the current study. The percentage of change in this enzyme’s activity at relapse was actually greater than the changes seen in either HPRT or PRPP.

Although PRPP is the cellular factor responsible for donating the ribose-phosphate group that is essential for conversion of MP to its active, nucleotide metabolite, its role in the emergence of clinical MP resistance has not been previously examined. Of interest, reduced PRPP levels were the most common biochemical change noted in the patients in the current study, although there was a large amount of interpatient variation in this parameter. Reduced PRPP levels have recently been reported to be present in an in vitro study characterizing a cell line selected for MP resistance (15). In addition, PRPP levels have been reported to be much lower in leukemic cells obtained directly from patients than PRPP levels in the L1210 cell line, suggesting that the availability of PRPP could be an important determinant of MP clinical activity (16). In the present study, PRPP levels fell in each of the three patients in whom a greater than 50% decrease in HPRT activity at relapse was documented. In contrast, when cell lines are selected for thiorpurine resistance and become HPRT deficient, an increase in PRPP levels has been reported (17, 18). It has been previously reported that the HPRT gene is located next to the PRPP synthetase gene on the X chromosome (19). The simultaneous decrease in both HPRT and PRPP at relapse in three patients raises the possibility that this change, resulting in decreased MP sensitivity, may have occurred at the genetic level.

Of the four patients in whom biochemical changes were demonstrated at relapse, all but one had a change in more than a single parameter. In two of the four patients, changes in all three biochemical parameters were documented at relapse. The apparently simultaneous appearance of decreased HPRT, decreased PRPP, and increased alkaline phosphatase occurring in the same patients at relapse would not have been predicted from in vitro MP studies done with cell lines. In the in vitro setting, cells selected for MP resistance have a single biochemical alteration accounting for the emergence of the resistant phenotype (4). Unlike the in vitro situation, clinical resistance to MP arises in patients treated with multiple chemotherapeutic agents, and therefore there may be a number of selective pressures operating on leukemic cells simultaneously. However, our results suggest that, in some patients receiving MP-containing chemotherapy on a chronic basis, several biochemical parameters may change within leukemic cells. If these changes are involved in the development of clinical resistance to MP, it is interest to note that even within the same patient alterations in more than one parameter may occur. Although it would have been of interest to measure the in vitro ability of leukemic cells obtained from patients to convert MP to the drug’s active metabolite, TIMP, a paucity of clinical material precluded the examination of this measurement of MP activation.

In the present study, only four of ten patients developed biochemical changes during MP-containing therapy that might have resulted in reduced MP activity. In addition, the large degree of interpatient variability in these biochemical values and the small number of patients in this study do not permit one to draw definitive conclusions regarding the development of clinical MP resistance and changes in the above biochemical parameters. Further biochemical studies (i.e., determination of Keq values for MP and PRPP for the enzyme, HPRT) may shed additional light on this problem. As patients with acute lymphoblastic leukemia are treated with multiple chemotherapeutic agents, it is unlikely that the development of resistance to any one particular drug used in leukemia therapy could account for most of the relapses seen in this disease. Moreover, other chemotherapeutic agents that the patients received may have affected the biochemical parameters measured (i.e., methotrexate effect on PRPP levels). The results of the present study, however, document the wide variation in HPRT, PRPP, and alkaline phosphatase in patients with ALL. In addition, this study shows that some patients treated chronically with MP-containing chemotherapy develop changes within their leukemic cells in one or more of these biochemical parameters that may be related to decreased MP activation. Whether these changes are involved in the development of clinical drug resistance requires further study.

REFERENCES


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