Mechanisms of Resistance to 6-Thiopurines in Human Leukemia

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SUMMARY

The activities of enzymes involved in the formation and catabolism of 6-thiopurine nucleotide were assayed in leukocytes of leukemic patients considered either susceptible or resistant to the 6-thiopurines. Altered ratios of the activities of hypoxanthine-guanine phosphoribosyltransferase (H-GPRT) and adenine phosphoribosyltransferase were noted in leukocytes from 3 of 11 resistant acute nonlymphocytic leukemic (ANLL) patients, whereas normal ratios were found in all 7 resistant acute lymphocytic leukemic patients assayed. Leukocytes from one of the resistant ANLL patients had a marked decrease in H-GPRT activity, while the remainder were relatively deficient, as defined by an adenine phosphoribosyltransferase/H-GPRT (A/H) ratio greater than the average ratio from leukocytes of untreated patients plus 3 standard deviation units. Particulate-bound alkaline phosphatase was significantly elevated in leukocytes from 2 of 11 resistant ANLL and 6 of 7 resistant acute lymphocytic leukemia patients. Little correlation between acid phosphatase levels of leukemic leukocytes and clinical status was noted. Increased alkaline phosphatase activity tended to occur in resistant patients with normal adenine phosphoribosyltransferase/H-GPRT ratios. The findings suggested that either a decrease in H-GPRT or an increase in alkaline phosphatase activities was responsible, at least in part, for insensitivity to 6-thiopurines in 5 of 11 ANLL and 6 of 7 acute lymphocytic leukemia patients.

INTRODUCTION

Although the phenomenon of resistance to purine analogs has been under intensive investigation for over a decade, the mechanisms by which human tumors become unresponsive to the 6-thiopurines remain unclear (4). In nonhuman systems, the most frequent cause of acquired insensitivity to purine analogs has been the loss or marked decrease of the activity of the anabolic enzyme H-GPRT4 which is required to convert these antimetabolites to the nucleotide form, a process necessary for tumor inhibition. However, enzymatic studies designed to test this possibility in man have indicated that this mechanism is not operative in most human leukemias (5, 14). In contrast, Kessel and Hall (8) reported that leukemic leukocytes isolated from patients sensitive to 6-MP retained a significantly greater amount of radioactivity from 6-MP to which they were exposed than did leukocytes from resistant patients. While these findings might be interpreted as being due to greater anabolic enzyme activity and intracellular retention of 6-thiopurine nucleotides by sensitive cells, an increase in the activity of analog nucleotide degrading enzymes in the resistant cells might also have accounted for the decreased retention. Resistance to several purine analogs has been ascribed to increased degradation of the active inhibitory analog nucleotide form (2, 3, 7, 19). Furthermore, Wolpert et al. (19) provided evidence that an increase in catabolic activity associated with resistance to 6-TG by a transplanted murine neoplasm was due to a particulate-bound alkaline phosphatase. This investigation represents an effort to expand upon previous work on the anabolic enzyme profile of leukemic leukocytes, and also includes a study of the phosphatase activities within these cells, the goal being a characterization of the role of anabolic and catabolic systems in determining the sensitivity of leukemic leukocytes of humans to 6-thiopurines.

MATERIALS AND METHODS

Adenine-8-14C (4.05 mCi/m mole), hypoxanthine-8-14C (5.32 mCi/m mole), and 6-MP-8-14C hydrate (2.48 mCi/m mole) were purchased from New England Nuclear, Boston, Mass. PRPP tetradsodium salt (Lot 42C 8631-9) and unlabeled purines were purchased from Sigma Chemical Company, St. Louis, Mo. Cellex D (anion exchange cellulose) was obtained from Bio-Rad Laboratories, Richmond, Calif. Dextron 100c (M.W. 135,000) was a product of Sigma Chemical Company, and Aquasol was obtained from New England Nuclear.

Patients at the Yale New Haven Medical Center with the diagnosis of active acute leukemia were studied. No initial selection was imposed other than the availability of either peripheral blood with a significant blast count (all but 2 of 25 samples had a blast content >40%) or the availability of...
adequate relapse bone marrow from the leukopenic patient (5 samples). Of the 30 samples from 29 patients (1 patient before and after thiopurine therapy), 11 were collected from patients with acute lymphocytic leukemia (4 untreated with thiopurines) and 19 with ANLL (8 with ANLL before thiopurine treatment). Patients were further divided into 2 groups on the basis of drug exposure. Twelve patients classified as untreated had not received any purine analog but may have been given other chemotherapy. Eighteen patients, classified as treated, had a history of receiving either 6-TG or 6-MP alone or in combination with other agents and had relapsed during or after the regimen.

Leukocytes were prepared free of contaminating erythrocytes as described previously (14). The supernatant obtained after centrifugation at 105,000 × g was used for assay of both H-GPRT and APRT activities. Both the supernatant fraction and the remaining pellet were used to measure acid and alkaline phosphatase activities. The pellet was suspended in 0.01 M Tris-HCl (pH 7.6) and homogenized in an Omnimixer before phosphatase assays. Protein was determined by the method of Lowry et al. (11). The standard phosphoribosyltransferase assay contained 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 1 mM PRPP tetrasodium, and a radioactive purine base in a total volume of 10 μl. The final concentrations of purine substrates were 0.47 mM hypoxanthine, 0.62 mM adenine, and 0.51 mM 6-MP. The reactions were initiated by the addition of enzyme (usually 40 to 80 μg protein). Control tubes in which PRPP was omitted were included routinely. Incubation lasted 30 min at 37° with adenine and hypoxanthine and lasted 1 hr with 6-MP. The reaction was terminated by the addition of 50 μl of 0.2 M EDTA, and the mixture was kept at 4° until it was subjected to chromatography. Separation of the nucleotide products from the substrates was accomplished with small DEAE columns as previously described (14). The formation of product was linear over the time interval measured and was linearly proportional to enzyme concentration. Phosphatase activities were determined at 25° with a Gilford thermostated recording spectrophotometer by measuring the initial rate of hydrolysis of p-nitrophenyl phosphate, using the change in absorbancy at 410 nm. Alkaline phosphatase was assayed in 0.05 M sodium bicarbonate buffer, pH 9.2, and the pH 5.8. The reaction mixtures contained 2.8 ml of the appropriate buffer and 10⁻³ M p-nitrophenyl phosphate; the reaction was initiated by addition of 0.2 ml of the enzyme either from the supernatant fraction or from the suspension of the sonically disrupted pellet, as described above. With the sonically disrupted suspension as the enzyme source, the reaction was allowed to continue for at least 20 min to overcome interference from turbidity, and the linear portion of the slope was taken for calculation of enzyme activity. One unit of activity is expressed as hydrolysis of 1 nmole of substrate per min under the assay conditions, assuming a molar absorbancy of p-nitrophenol in the buffers used of 1.7 × 10⁴/mole/liter/cm. Specific activity refers to nmol Pₐ per min per mg of protein.

RESULTS

Anabolic Enzymes. A study of the nucleotide-forming capacity of leukemic cells, enlarging upon previously published series (5, 14), was undertaken to determine the relative contribution of decreased anabolic activity in leukocytes resistant to the 6-thiopurines. The findings obtained with leukemic leukocytes from purine analog-treated and untreated groups of patients are illustrated in Table 1 and Chart 1. A wide scatter of values occurred in both untreated and treated categories, and no statistically significant difference existed between the mean values of the 2 groups; however, it should be noted that there is only 1 patient with a phosphoribosyltransferase activity (6-MP used as the substrate) less than 50 nmol per mg protein per hr in the group that did not receive a purine analog, while 6 samples in the treated population had lower enzyme activity. It has been found useful to compare, in cell-free extracts from leukocytes of individual patients, the activity of H-GPRT, which anabolizes 6-TG and 6-MP to their active nucleotide forms, with that of APRT, a supernatant enzyme that converts adenine to adenosine 5'-phosphate, in order to assess changes in the relative potential for formation of purine nucleotides by the cells under study (6). The means of the A/H ratio of untreated and treated groups were equivalent; in particular, the values of the untreated population formed a tight cluster about the mean. The mean for the untreated group, 2.71 units, plus 3 S.D. units (arbitrarily chosen) was equal to 4.60 units. Three treated ANLL patients exceeded this value. The patient with the lowest H-GPRT activity, with either hypoxanthine or 6-MP as the substrate, had an A/H value of 101; this individual is a clear example of H-GPRT deficiency in purine antimetabolite-resistant leukemia.

Table 1: Anabolic and catabolic leukocyte enzyme activities in treated and untreated patients with acute leukemia

<table>
<thead>
<tr>
<th></th>
<th>Phosphoribosyltransferase activity (nmole nucleotide/mg protein/hr)</th>
<th>Phosphatase activity (nmole Pₐ/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adenine</td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>No. of patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 untreated</td>
<td>1007 ± 513*</td>
<td>372 ± 196</td>
</tr>
<tr>
<td>18 treated</td>
<td>644 ± 392</td>
<td>234 ± 141</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

* Excluding 1 patient with a particulate-bound alkaline phosphatase level of 12.8 nmol Pₐ per mg protein per min.

* Excluding 1 patient with an A/H ratio of 100.9.
treated leukemia. As described previously (14), enzymic activities of leukocytes from all patients with A/H ratios greater than 4.60 units were evaluated kinetically to determine whether mutant enzymes with altered affinity for the substrate PRPP could account for the relatively low H-GPRT activity of these patients. No enzymatic defects similar to those reported previously were observed (1, 12, 14).

Catabolic Enzymes. The activities of phosphohydrolase enzymes that might be expected to affect the cellular concentration of analog nucleotide are listed in Table 1 and Chart 2. Phosphatase activity of the supernatant fraction after centrifugation of cell sonic extracts at 105,000 × g for 1 hr, and of the residual particulate fraction, was measured at both acid and alkaline pH values. Particulate-bound alkaline phosphatase was the only measured enzyme that showed a clear elevation in leukocytes from some of the treated patients. A breakdown by cell type demonstrated that all 4 patients with acute lymphocytic leukemia that were never treated with purine antimetabolites had particulate alkaline phosphatase activity less than 1 nmole per mg protein per min, while 6 of 7 patients previously treated with these agents had higher levels of activity. Among patients with ANLL, the distinction was less clear. Of the 8 untreated patients, 1 had the highest value for particulate-bound leukocyte alkaline phosphatase recorded in our laboratory to date. This patient was treated for 21 days with a full course of cytosine arabinoside (1-β-D-
arabinofuranosylcytosine) and 6-TG (100 mg/sq mm of each drug every 12 hr) without remission. The remainder of the untreated patients with ANLL had little or no particulate-bound leukocyte alkaline phosphatase activity. Of the 11 treated patients with ANLL, 2 had high, 2 intermediate, and the remainder no particulate-bound alkaline phosphatase activity. No correlation between patient status and the activities of either soluble alkaline phosphatase or soluble or particulate acid phosphatase was apparent.

DISCUSSION

The levels of the enzyme H-GPRT in leukemic leukocytes of patients deemed susceptible or resistant to the antileukemic activity of 6-MP and 6-TG are in general agreement with previously published results (5, 14). To date, severe deficiency of this enzyme has been reported in human leukemia by Davidson and Winter (5) in 1 of 14 patients, by Rosman and Williams (14) in 1 of 8 patients, and in this investigation in 1 of 18 treated patients. However, use of the A/H ratio discriminator has allowed the detection of the possible presence of a moderate anabolic enzyme deficiency in an additional 6 resistant cases [i.e., in 3 additional patients in the study by Davidson and Winter (5), in 1 other case in the series of Rosman and Williams (14), and in 2 additional individuals in this study]. Decreases in H-GPRT activity associated with insensitivity to purine analogs have occurred only in patients with ANLL. Thus, in this disease, severe H-GPRT deficiency may account for 12% of resistant cases, while moderate enzyme deficiency may be responsible for an additional 23%. Loss of analog nucleotide-forming potential was not implicated in the mechanism of resistance to 6-thiopurines in acute lymphocytic leukemia to a significant extent.

The relatively low frequency of attainment of H-GPRT mutants in 6-thiopurine-resistant leukemic leukocytes of humans compared with murine neoplasms and bacterial systems (4), may be a consequence of little or no de novo biosynthesis of purine nucleotides by the leukemic leukocytes (15, 17), coupled with major dependence by these cells on the utilization of preformed purines for the formation of coenzymes and nucleic acids (9, 13). Should such mechanisms be operative, leukemic leukocytes from patients achieving insensitivity to 6-TG and 6-MP by lowering H-GPRT activity would be expected to be highly dependent upon preformed adenine utilization for survival. This population of neoplastic cells should, therefore, be susceptible to cytotoxic analogs of adenine and, for example, to 5(4')-amino-4(5)-thiomidazole carboxamide which, following expected activation by APRT to the nucleotide level and ring closure, would generate 6-thioinosinic acid by an alternate route. This latter possibility is under consideration by our laboratory.

A change in catabolic potential for nucleotide degradation by leukemic cells of humans (i.e., increased phosphohydrolase activity), as described for a transplanted murine 6-thiopurine-resistant neoplasm (3, 19), theoretically appeared to be a less difficult alteration for these cells to accomplish resistance to either 6-MP or 6-TG. In support of this concept, particulate-bound alkaline phosphatase activity was elevated in leukocytes from 6 of 7 patients with acute lymphocytic leukemia deemed resistant to purine antimetabolites, while most untreated patients had negligible activity (Chart 2). Of the patients with ANLL, 2 had high values for particulate alkaline phosphatase activity. Leukocytes from 5 of 11 patients with ANLL had either elevated particulate alkaline phosphatase activity or decreased H-GPRT activity associated with clinical resistance. No correlation between the clinical status of patients and the activities of particulate-bound acid phosphatase or of soluble acid or alkaline phosphohydrolases was evident. Of the untreated patients, 1 showed an extremely high level of particulate alkaline phosphatase activity and a 2nd had an intermediate level of activity (0.91 nmole P_I per mg per min). However, the predictive value of elevated particulate alkaline phosphatase levels in untreated patients as to their subsequent response to chemotherapy with purine analogs is unclear from this study. Although the patient with ANLL with a particulate alkaline phosphatase activity of 12.8 nmoles P_I per mg protein per min proved resistant to a rather extraordinary course of chemotherapy with cytosine arabinoside and 6-TG, as described above, and the patient with acute lymphocytic leukemia with an activity of 0.91 nmole P_I per mg protein per min relapsed after 2 months on the St. Jude regimen (16), there was an insufficient number of responders in our untreated group and an insufficient number of total untreated patients with elevated phosphatase levels to make the response data meaningful. Unfortunately, in this period of multidrug therapy, when the 6-thiopurines are no longer used to induce remission in acute lymphocytic leukemia, a prospective study would be extremely difficult to carry out. Nevertheless, it appears that in patients with acute lymphocytic leukemia, an increase in the capability of leukemic leukocytes to degrade analog nucleotide may be, at least in part, responsible for the attainment of insensitivity to the 6-thiopurines. The development of an effective inhibitor of alkaline phosphatase to be used in combination with 6-MP or 6-TG may, therefore, be important in restoring the sensitivity of such resistant cells to these agents. It appears that promising leads in this area are represented by the findings of our laboratory that derivatives of α-(N')-heterocyclic carboxaldehyde thiosemicarbazones (10) and tetramisole (18) are potent inhibitors of alkaline phosphatase isolated from the murine neoplastic model system, Sarcoma 180/TG.

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