Retention of 6-Mercaptopurine Derivatives by Intact Cells as an Index of Drug Response in Human and Murine Leukemias

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Summary

Human and animal leukemias, varying widely in responsiveness to 6-mercaptopurine, were examined to seek determinants of drug sensitivity. Drug-responsive rodent leukemias showed a comparatively high capacity for in vitro conversion of 6-mercaptopurine into nondiffusible cellular metabolites, predominantly drug nucleotides. A similar study of human leukemia cells indicated that the in vitro capacity for 6-mercaptopurine anabolism can also be a useful predictive measure of drug responsiveness in man.

Introduction

The present state of knowledge concerning the drug 6-MP does not permit the precise localization of a single primary site of drug action. The drug is known to interfere with purine nucleotide biosynthesis and interconversions of purine nucleotides, which could account for the antineoplastic action of 6-MP; the drug also has a profound inhibitory effect on the immunosuppressive system.

Prior conversion to nucleotide derivatives is believed to be a requisite for 6-MP action. Various workers have reported that the enzyme IMP-pyrophosphorylase catalyzes conversion of 6-MP into 6-TIMP (5, 8). The conversion of 6-MP into 6-TXMP (2, 13), 6-methylmercaptopurine ribotide (1), and several other metabolites (6), including nucleoside di- and triphosphates (19) has been reported. The contribution of these drug metabolites to the total pharmacologic effects of 6-MP is unknown.

Previous studies on animal tumors have shown that development of resistance to 6-MP usually involves loss of IMP-pyrophosphorylase activity in drug-resistant cells (4). In human leukemic cells, however, 6-MP resistance was not uniformly associated with decreased IMP-pyrophosphorylase levels (7), nor was there a consistent correlation between drug responsiveness and inhibition of purine nucleotide biosynthesis by 6-MP (7, 18).

The present study is part of a program designed to provide in vitro determinants of response of human leukemias to antitumor agents. Determinants of response of drug-sensitive and -resistant animal leukemias to 6-MP were first sought, and the relevance of these factors to the human leukemia situation was assessed.

Materials and Methods

Murine leukemias were supplied by Mr. I. Wodinsky, Arthur D. Little, Inc., Cambridge, Massachusetts. Sources of tumor lines (16) and methods of isolation of cells (12) have been described. Human leukocytes were isolated (15) and separated into different cell types (11). In the data presented here, cells from patients with chronic lymphocytic leukemias were 90-100% small lymphocytes, cells from patients with chronic myelogenous leukemias were 70-95% immature granulocytes, and cells from patients with acute leukemias were mainly (80-100%) blast forms.

Bone marrow specimens, provided by the clinical staff of the Children's Cancer Research Foundation, were collected in tubes containing EDTA (2 mg/ml), chilled to 0°C. Contaminating erythrocytes were lysed by dilution with 3 volumes of water at 0°C. After 30 sec, isotonicity was restored by addition of 1 volume of 3.6% NaCl. The unbroken cells were collected by centrifugation at 500 x g for 10 minutes, washed once with 0.9% NaCl by resuspension, and collected by centrifugation as before. The final preparations contained 75% leukocyte precursors. Human leukemia cells, grown in culture, were provided by Dr. George Moore, Roswell Park Memorial Institute, Buffalo, N. Y.

The 6-mercaptopurine-8-14C (3 mc/mmole) was purchased from New England Nuclear Corp. and was stored in solution at pH 7, usually at -20°C. Purity of preparations was determined by ultraviolet absorption spectra (9) and by chromatography (2).

For in vitro studies, murine leukemia cells were suspended in a buffered-salt solution (13) to yield a 5% cell suspension. Human leukocyte suspensions (5%) were prepared in a slightly different buffer, containing 25% dialyzed calf serum (11).

Cell suspensions were distributed in 200-μl aliquots in 10 x 30 mm siliconized glass tubes, labeled 6-MP was added, and the tubes were shaken for specified intervals. Incubations were terminated by chilling the tubes, followed by centrifugation at 500 x g for 30 sec. Details of this procedure have been...
reported (10). The pellets obtained were used for measurement of total drug uptake, after suitable correction for trapped extracellular fluid (13). To measure the intracellular pool of nondiffusible drug metabolites, the pellets were resuspended in 400 μl of fresh incubation medium and warmed to 37°C for 5 minutes to allow the loss of the rapidly diffusible pool of radioactivity (10). The cells were then collected by centrifugation as before.

To measure cellular radioactivity, pellets were uniformly suspended in 250 μl of 0.9% NaCl, and a 200-μl portion taken for liquid scintillation counting. Data are reported in terms of μmoles of radioactive drug equivalents per kg of cells (wet weight) per minute. Wright-stained preparations of all cell samples were examined to yield data on cell types present. Drug metabolites in cell extracts were characterized as described in Reference 14.

Data on the responsiveness of animal leukemias to 6-MP were provided by Mr. I. Wodinsky.4 Drug resistance in human leukemias refers to the inability of 6-MP to produce partial marrow remissions as defined by the Acute Leukemia Cooperative Group B of the National Cancer Institute (3). The drug was given parenterally or orally, 2–5 mg/kg daily alone, or in combination with 1 mg/kg of oral Prednisone.

RESULTS

Studies with Animal Leukemias. Uptake of 6-MP by all cell lines tested was a rapid process, yielding a cell/medium distribution of approximately one, regardless of incubation temperature, over a wide range of drug concentrations (Table 1). These experiments were carried out using incubation times of one minute, the shortest time period attainable under present conditions. In the L1210/MP cell line, in which 6-MP is converted to the nucleotide level at a slow rate, the distribution ratio was not increased by prolonged incubations (60–120 minutes). In other cell lines, incubations for more than 3–5 minutes, at 37°C, led to apparent drug distribution ratios greater than one; this was caused by conversion of 6-MP to nondiffusible derivatives which accumulated within cells. When cells were incubated with labeled 6-MP, and then resuspended in fresh medium at 37°C, the free drug was rapidly lost (τ < 1 minute), but the nondiffusible labeled products (mainly 6-TIMP) remained.5 The relative rate of drug conversion to such products varied among the different cell types tested and was related to drug-promoted survival of tumor-bearing animals (Chart 1). The incubation time routinely used for these studies was 15 minutes at 37°C. We found that the rate of drug conversion to nondiffusible metabolites remained constant for at least 30 minutes. The standard time for extraction of the cells was 5 minutes at 37°C. Chromatographic examination of cell extracts showed that no free 6-MP remained in the cells after this treatment.

Table 1

<table>
<thead>
<tr>
<th>Drug level in medium (mM)</th>
<th>L1210/6-MP</th>
<th>L1210</th>
<th>Dunning/Schmidt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>1.02</td>
<td>1.06</td>
<td>1.10</td>
</tr>
<tr>
<td>0.1</td>
<td>0.98</td>
<td>1.03</td>
<td>1.05</td>
</tr>
<tr>
<td>1.0</td>
<td>0.95</td>
<td>1.02</td>
<td>0.96</td>
</tr>
</tbody>
</table>

*Distribution of 6-mercaptopurine (6-MP) between cell water and medium in animal tumors.

5The ratio of cellular level (reached after 1 minute at 37°C) to extracellular level. These values represent averages of 3 measurements which differed by not more than ±10%. The values shown here were not significantly altered by lowering incubation temperatures to 10°C.

Studies with Human Leukemias. No differences were found when the permeabilities of different cell types to 6-MP were measured (Table 2), but the rate of cellular conversion of the drug to nondiffusible products varied widely (Chart 2) with 6-TIMP predominating.5 Of the normal cell types, this conversion rate was highest in marrow cells and lowest in small lymphocytes. When cell preparations from patients with leukemia were examined, the conversion rate was found to be uniformly low in small lymphocytes from chronic lymphocytic leukemias. A wide range of conversion rates was found in the other leukemias: patients with disease found clinically

4Data on drug-promoted survival of tumor-bearing animals were provided by Mr. I. Wodinsky, Arthur D. Little, Inc. Animals were treated with 40 mg/kg of 6-MP from Day 1 to Day 10 following intraperitoneal inoculation with 10⁶ tumor cells.

5Chromatographic studies (2, 14) indicated the presence of at least five components; the major component migrated with an authentic sample of 6-TIMP.
The precise mode of action of 6-MP is not yet clearly understood, nor have all pathways of drug metabolism been determined. We have therefore attempted to ascertain whether a relation exists between 6-MP conversion to nondiffusible derivatives (presumably drug nucleotides and drug, or drug derivatives, bound to cell components) and drug response. Formation of nondiffusible drug products had been demonstrated in normal leukocytes (11).

In the present study, the incubation of freshly isolated ascitic tumor cells with 0.1 mM 6-mercaptopurine-8-14C for 15 minutes at 37°C produced intracellular drug derivatives which, unlike free 6-MP, could not be readily washed from the cells. The rate of this conversion was correlated with responsiveness to 6-MP conversion in vivo. Incubation conditions found relevant for animal tumor studies were then employed with human leukocyte preparations.

It is noteworthy that small lymphocytes (normal or from patients with chronic lymphocytic leukemia) showed the sensitive to 6-MP demonstrated comparatively high rates of drug conversion to nondiffusible products; drug resistance was associated with low conversion values.

Leukocytes from one patient with chronic myelogenous leukemia were tested both before and after development of clinical resistance to 6-MP. The sample marked a (Chart 2) was obtained before drug therapy was initiated. These cells (mainly promyelocytes and promyelocytes) showed a comparatively high capacity for drug conversion to nondiffusible products in vitro. After a remission induced by 6-MP, the patient later relapsed and was found to be unresponsive to the drug. Sample b was obtained at that time; the drug conversion rate had decreased by 50%.

Cell preparation c (Chart 2) was obtained from another patient with chronic myelogenous leukemia. At the time of sampling, a leukocyte count of 144,000 was found (75% immature granulocytes). Radiation therapy (1175 R over a 2-month period) was initiated. At the end of this time, abnormal cells had disappeared from the circulating blood, and the level of 6-MP conversion had returned to a value comparable to that found in leukocytes obtained from normal donors (0.5 μmoles/kg cells/min). At a later time, immature granulocytes again were found in the circulating blood, the rate of 6-MP conversion was elevated, and the patient was clinically sensitive to 6-MP.

Except for the examples noted above, the reported insensitivity to 6-MP was not the result of prior drug therapy. An additional group of pediatric patients with acute lymphocytic leukemias were tested; these patients had one or more remissions on therapeutic programs which included 6-MP, but were unresponsive to such programs at the time of testing. Corresponding values for 6-MP conversion by this group of 12 patients ranged from 0.2-0.6 μmoles/kg cells/min.

For comparison, rates of 6-MP conversion to nondiffusible products by a group of cultured human leukemias is also shown in Chart 2. The results are typical of other such cell lines we have examined; the conversion rate is higher than that generally found in cells freshly isolated from circulating blood.

**DISCUSSION**

The precise mode of action of 6-MP is not yet clearly understood, nor have all pathways of drug metabolism been determined. We have therefore attempted to ascertain whether a relation exists between 6-MP conversion to nondiffusible derivatives (presumably drug nucleotides and drug, or drug derivatives, bound to cell components) and drug response. Formation of nondiffusible drug products had been demonstrated in normal leukocytes (11).

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It is noteworthy that small lymphocytes (normal or from patients with chronic lymphocytic leukemia) showed the
The lowest capacity for 6-MP conversion into the nondiffusible fraction. Only a few samples of cells from patients with other types of leukemias showed such a limited drug conversion. Mature granulocytes and marrow cell preparations showed a somewhat higher capacity for drug conversion than did lymphocytes. In contrast, acute and chronic myelogenous and acute lymphocytic leukemia cells exhibited a wide range of conversion rates.

The data shown in Chart 2 indicate a relationship between capacity for 6-MP conversion in vitro by isolated leukemia cells and the responsiveness of patients to the drug. In contrast to animal tumors, human leukemias present a variety of variables which cannot be controlled and the results should be interpreted accordingly. Only a limited number of sequential studies on individual patients were available; an example of drug-induced resistance to 6-MP was clearly associated with a significant decrease in cell capacity for 6-MP anabolism. In another patient, the replacement of chronic myelogenous granulocytes with normal cell types, following roentgen therapy, was accompanied by a fall in the capacity for 6-MP conversion to nondiffusible products by circulating leukocytes.

A group of cultured cells, derived from the circulating leukocyte population of patients with acute leukemias, showed a generally high capacity for 6-MP conversion to the nondiffusible fraction. Sensitivity of these cells to 6-MP in culture is unknown, but the data suggest that drug-sensitive cell types were selected during the procedures.

The data obtained here must be compared with observations on the levels of enzymes involved in the conversion of 6-MP by drug-sensitive and resistant cell types to proposed "active" forms. The observations summarized by Brockman (4) indicate that a common characteristic of most 6-MP resistant cells is the lack of capacity to form 6-TIMP via IMP- pyrophosphorylase. In the present assay system, this would be revealed as a decreased capacity for intact cells to convert 6-MP to nondiffusible (nucleotide) derivatives. [Enhanced destruction of 6-MP might also be a factor in certain examples of drug resistance (17).] Leukocyte homogenates from patients with leukemias resistant to 6-MP did not generally show impaired IMP-pyrophosphorylase (7).

The procedure described here has proved useful in predicting responsiveness of patients with leukemia to prospective 6-MP chemotherapy. A characterization of the nondiffusible products of 6-MP, formed by drug-sensitive but not drug resistant cell types, might result in the design of a simple enzymatic assay applicable to cell extracts.

REFERENCES

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