Uptake and Phosphorylation of Cytosine Arabinoside by Normal and Leukemic Human Blood Cells in Vitro

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SUMMARY

Uptake and subsequent phosphorylation of cytosine arabinoside by normal and leukemic human leukocytes, and by normal platelets and erythrocytes, were measured in vitro. A very rapid uptake of the drug was found, even at 10\(^{°}\)C. A cell-medium distribution ratio of 1 was achieved over a wide range of external drug concentrations. Capacity for drug phosphorylation, which varied widely among the different cell types, was highest in mature lymphocytes and some myeloblasts and lymphoblasts, lower in granulocytes, erythrocytes and platelets, and lowest in circulating lymphoblasts and myeloblasts from patients with leukemias resistant to cytosine arabinoside.

INTRODUCTION

ara-C\(^3\) is a synthetic nucleoside which inhibits growth of certain animal neoplasms (7, 8, 25) and has been useful in the treatment of human leukemias (3, 10, 24). A requisite for the action of this drug is prior conversion to nucleotides (4, 5, 22). Mechanisms of ara-C action, based on interference with ribotide reduction (4) and upon ara-C incorporation into cell DNA (5, 6, 23), have been proposed. Recent findings have argued against the former hypothesis (18), and competition between ara-CTP and deoxycytidine triphosphate for DNA polymerase (1, 9) seems a major site of drug action. Extensive deamination of ara-C in man has been found (2, 6). The product, ara-U, is of unknown pharmacologic activity.

Response to ara-C by animal tumors was related to their capacity for ara-C phosphorylation (5, 16). There was no evidence to suggest barriers to the uptake of the drug in ara-C-resistant animal leukemias. The present study was designed to investigate ara-C uptake and phosphorylation by various types of human blood cells and to examine ara-C phosphorylation as a possible determinant of drug response in human leukemias. A preliminary account of this report has been presented (11).

MATERIALS AND METHODS

Labeled Nucleosides. ara-C, tritiated mainly in the pyrimidine ring (3 c/m mole), was provided by the Cancer Chemotherapy National Service Center. The drug was purified by descending paper chromatography (Whatman No. 1) for 20 hours, using n-butanol:water:formic acid (77:13:10). This system can resolve cytosine, ara-C, and ara-U; nucleotides remain at the origin. Appropriate markers were used to aid in location of these compounds under ultraviolet light.

Labeled ara-U was prepared as follows: 5 microliters of a solution of 500 mM ara-C\(^{3}\)H were mixed with 1 \(\mu\)l of 6 N NaNO\(_2\) and 1 \(\mu\)l of glacial acetic acid was added. The mixture was heated to 60\(^°\)C for 30 minutes in a stoppered tube; an additional 1 \(\mu\)l of NaNO\(_2\) solution was added and the heating continued for 30 minutes. The mixture was then subjected to paper chromatography on Whatman No. 1 paper as described above. The region containing labeled ara-U was cut out, eluted with water, and this solution concentrated in vacuo and diluted to 5 mM concentration. The ara-U chromatographed with a genuine sample of the compound in several other solvent systems (5). The yield, based on ara-C, was 80—90%.

Nonlabeled ara-C and ara-U were provided by Dr. Charles Smith, and ara-CMP was provided by Dr. Gary Gray, both men being affiliated with the Upjohn Company, Kalamazoo, Michigan.

Isolation of Blood Cells. Methods used for collection and isolation of lymphocytes and granulocytes and of leukemic cells from peripheral blood have been described (13, 14). Marrow samples (1—5 ml) were collected in 3 ml of 0.9% NaCl containing 35 mg of EDTA. Erythrocytes were lysed by dilution with 3 volumes of water at 0\(^°\)C, and, after 30 seconds, 1 volume of 3.6% NaCl was added to restore isotonicity. The marrow cells were concentrated by centrifugation for 10 minutes at 500 \(\times\) g, washed twice by resuspension in 0.9% NaCl and centrifugation. Erythrocytes were collected from normal donors, washed three times by suspension into 20 volumes of 0.9% NaCl; each time the cells were then collected by centrifugation at 500 \(\times\) g for 10 minutes. Platelets were obtained from whole blood, using citrate as an anticoagulant. After removal of leukocytes...
Peripheral leukocytes were fractionated (19, 20) to yield preparations containing 95% lymphocytes or 95–100% granulocytes. Erythrocyte and platelet preparations were not contaminated by other cell types. Marrow aspirations, after treatment described above, contained approximately equal numbers of erythroid and myeloid precursors. Peripheral cells from patients with chronic lymphocytic leukemia were 85–100% small lymphocytes. Cells from patients with chronic myelogenous leukemia were 70–100% immature granulocytes, and those from patients with acute leukemias were 50–100% blast forms. Cell viability was estimated by ability of preparations to exclude 0.025% trypan blue; less than 10% of the cells took up the dye. Wright-stained slides of all cell preparations were examined to determine relative proportions of different cell types.

Incubation of Blood Cells. Leukocytes, marrow cells, and erythrocytes were suspended in 10 volumes of a medium containing the following: 65 mM NaCl, 15 mM KCl, 8 mM CaCl₂, and 62 mM TES buffer at pH 7.2, all in 25% dialyzed calf serum (13, 14). Platelets were suspended in 10 volumes of medium in which an equimolar amount of sodium citrate was substituted for calcium chloride.

Studies of ara-C Uptake. Uptake of ara-C-³H was measured by incubation of leukocyte suspensions with 0.05–25.0 mM drug for 3 minutes at 10°C. The cells were collected by centrifugation for 30 seconds at 150 × g, the supernatant fluid was discarded, and the cell pellets were blotted dry with wedge-shaped filter paper. To correct for extracellular fluid trapped in the cell pellets, a similar experiment was carried out using 0.01 M (final concentration) labeled sulfate. We have found blood cells to be equally impermeable to sulfate and inulin; sulfate has the advantage of stability to storage. This correction amounted to 30–40% of the wet weight of the cell pellets. The total water space of cell pellets was measured by incubations in medium containing tritiated water. These experiments indicated that 35–40% of the wet weight of the cell pellets represented intracellular water. These data are in agreement with data previously reported (13, 21) for normal human leukocytes.

Uptake of ara-C by erythrocytes could not be determined by the method described above because of difficulty in obtaining compact cell pellets. To measure permeability of erythrocytes to ara-C, 500 μl of incubation medium containing labeled ara-C or tritiated water was mixed with 500 μl of a concentrated erythrocyte suspension (hematocrit = 70). After 3 minutes at 10°C, the cells were sedimented by centrifugation at 500 × g for 10 minutes, and a portion of the supernatant fluid was removed for determination of radioactivity. The total water space (intracellular + extracellular) permeable to the isotope was estimated by measuring the total dilution of labeled material caused by addition of the cell suspension.

Studies of ara-C Phosphorylation. To estimate relative capacity for conversion of ara-C (and ara-U) to nucleotides, blood cells were incubated with the labeled compounds for 15 minutes at 37°C, then collected by centrifugation for 30 seconds at 150 × g and the supernatant fluid discarded. The cells were then resuspended in 20 volumes of fresh medium and held at 37°C for 5 minutes to wash out free nucleoside. Under these conditions, nucleotides were not lost (15, 16). The cells were collected by centrifugation as before. Erythrocytes were given a further 4 washings to completely remove extracellular radioactivity from the cell mass. The cells were finally suspended in 0.9% NaCl. Generally, pellets of 15–20 mg were obtained, and these were suspended in 250 μl of the NaCl solution. A 200-μl portion of this suspension was taken for determination of intracellular, labeled nucleotides by liquid scintillation technics (15). For erythrocytes a slightly different method was used; the 200-μl cell suspension was mixed with 200 μl of 70% HClO₄; 400 μl of 30% H₂O₂ was added, and the mixture was heated at 70°C for 30 minutes to effect complete digestion. Radioactivity was subsequently determined by liquid scintillation counting as specified in Ref. 17.

Characterization of Products. Cell pellets, after incubation with labeled ara-C, were uniformly mixed with 5 volumes of 0.5 M HClO₄ for 15 minutes at 0°C. The debris was removed by centrifugation, and the supernatant fluid was adjusted to pH 7 with 1 M KOH. The neutralized solution was kept at 0°C for 30 minutes to complete precipitation of KClO₄, which was removed by centrifugation. Paper chromatographic analysis of the fluid was carried out on Whatman No. 1 paper using isobutyrate-NH₃ (16) or the butanol-water-formic acid solvent described above. These solvents readily separate ara-C from drug nucleotides. Radioactivity in regions of the chromatograms corresponding to marker ara-C and nucleotides was measured by liquid scintillation counting. Recovery of 90% of applied radioactivity was generally observed, although counting efficiencies were low (2–5%).

RESULTS

Uptake of ara-C. There were no apparent barriers to ara-C uptake in erythrocytes (Table 1); these cells were equally permeable to ara-C and tritiated water. Normal and leukemic leukocytes were also freely permeable to the drug (Table 2). Rapid cell/medium equilibrium was attained within 3 minutes of incubation at 10°C. Although the temperature was kept low to minimize phosphorylation, part of the observed uptake (10–20%) represented intracellular conversion of ara-C to non-diffusible nucleotides. This accounted for the apparent concentration of ara-C sometimes observed.

Phosphorylation of ara-C by Blood Cell Types in Vitro. There were major differences in the capacity of normal granulocytes and lymphocytes for conversion of ara-C to nucleotides in vitro (Chart 1), with the lymphocytes showing far greater capacity for such conversion. The cellular phosphorylation processes were saturated with substrate only at drug levels far in excess of those encountered during usual drug therapy, as will be discussed.

The phosphorylation of ara-C by all cell types was linear for at least 30 minutes and was decreased when the incubation temperature was lowered (Chart 2). A comparison of the relative capacities for conversion of ara-C to nucleotides by blood cells from normal donors (marrow precursors, granulocytes, lymphocytes, erythrocytes, and platelets) and by leukemic cells is shown in Chart 3. In some cases, the response of donor
In a previous study (16), responsiveness of animal leukemias to ara-C was related to the capacity of the different cell lines for drug phosphorylation in vitro. No barriers to drug uptake were found, even in a subline of L1210 made ara-C-resistant by exposure to the drug. (In other studies to be reported elsewhere, we found that ara-C uptake in a mammalian cell line lacking subsequent drug phosphorylation was mediated, but apparently not saturable, at 37°C.) In the present study, all human cell types tested were readily permeable to ara-C at 10°C to drug levels employed in these studies, as well as at considerably higher levels. Permeability studies were carried out at 10°C to minimize drug phosphorylation; at 37°C almost no free cellular ara-C remained after the isolation procedure described above. Apparently, the capacity of most cell types for ara-C phosphorylation at 37°C is sufficient to convert all of the free intracellular compound to nucleotides during the time required to collect the cells. Since ara-C readily penetrates all cell types tested here, even at 10°C, it seems unlikely that barriers to uptake would exist in drug-resistant cells at 37°C.

The conditions selected for the present study were previously employed in the work cited above (16). Although the drug phosphorylation process was far from saturated with substrate at the levels routinely employed, these levels reflect the approximate serum levels we estimate to be initially achieved during clinical drug therapy using direct 10 mg/kg i.v. pulse injections of the drug. The incubation times used were found relevant for mouse leukemia studies (16) and are believed appropriate for study of a drug which is rapidly lost from the circulation (6).

### DISCUSSION

<table>
<thead>
<tr>
<th>Radioactive compound</th>
<th>Initial radioactivity (cpm/µl)</th>
<th>Final radioactivity (cpm/µl)</th>
<th>Dilutiona (µl)</th>
</tr>
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<tbody>
<tr>
<td>ara-C</td>
<td>320</td>
<td>178</td>
<td>899</td>
</tr>
<tr>
<td>THO</td>
<td>780</td>
<td>433</td>
<td>901</td>
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</table>

Permeability of erythrocytes to cytosine arabinoside (ara-C) and tritiated water (THO). One volume (500 µl) of medium containing ara-C-3H or THO was diluted with one volume of a suspension of erythrocytes (hematocrit = 70). After 5 minutes at 10°C, the cells were collected by centrifugation, and the radioactivity in an aliquot of the supernatant fluid was measured.

### Table 2

<table>
<thead>
<tr>
<th>Drug level (mM)</th>
<th>Cell/medium drug distribution ratio</th>
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<tbody>
<tr>
<td></td>
<td>Lymphs</td>
</tr>
<tr>
<td>0.05</td>
<td>1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>5.0</td>
<td>0.94</td>
</tr>
<tr>
<td>25</td>
<td>0.96</td>
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</table>

Uptake of cytosine arabinoside (ara-C) by human leukocytes. Cells were incubated for 3 minutes at 10°C in medium containing specified levels of ara-C-3H. The intracellular ara-C level was determined using unwashed cell pellets, with suitable corrections for trapped extracellular fluid and for cell space not permeable to tritiated water. Sources of cells are described in the text. These data represent values for one experiment; repetitions yielded results which did not differ from these by more than ±5%. Chromatographic studies indicated that at least 80% of the intracellular radioactivity represented free ara-C.

Table 1

| Drug level (mM) | Uptake of cytosine arabinoside (ara-C) by normal lymphocytes. Cells were incubated for 3 minutes at 10°C in medium containing ara-C-3H. The intracellular ara-C level was determined using unwashed cell pellets, with suitable corrections for trapped extracellular fluid and for cell space not permeable to tritiated water. Sources of cells are described in the text. These data represent values for one experiment; repetitions yielded results which did not differ from these by more than ±5%. Chromatographic studies indicated that at least 80% of the intracellular radioactivity represented free ara-C.

Table 2

Patients to ara-C was known. Certain leukemic cell preparations were later shown to be drug-sensitive, as indicated by ara-C-induced remissions in donor patients. Other cell samples were from patients known to have ara-C-resistant leukemias. All other leukemia cell samples were from patients never exposed to the drug, with unknown sensitivity to ara-C.

The temperature sensitivity of ara-C phosphorylation by two myeloblast preparations is shown in Chart 2. The AML1 cells showed a relatively high capacity for ara-C phosphorylation; the AML2 cells were from a patient with ara-C-resistant leukemia. At the ara-C level routinely employed (0.05 mM), phosphorylation proceeded at a constant rate in both myeloblast preparations for 30 minutes. These data are typical of findings obtained with other normal and leukemic leukocyte preparations.

Phosphorylation of ara-U. At equimolar levels, ara-U was converted to nucleotides by most cell preparations at 5—10% of the rate found for ara-C (Table 3). Although the phosphorylation of ara-U did not vary widely among the different cell types tested, the ratio of ara-U to ara-C phosphorylation was highest in normal granulocytes. These data have implications in assessing the probable effect of ara-C deaminase found in some cell preparations. This will be considered more fully below.

In recent studies, we have been unable to demonstrate phosphorylation of ara-U in cell-free extracts of normal or leukemic leukocytes.

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Table 3

<table>
<thead>
<tr>
<th>Cell type</th>
<th>ara-U-phosphorylation (amoles/kg cells/minute)</th>
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</thead>
<tbody>
<tr>
<td>Normal lymphs (8)</td>
<td>0.15—0.2</td>
</tr>
<tr>
<td>Normal polys (5)</td>
<td>0.19—0.26</td>
</tr>
<tr>
<td>CLL (4)</td>
<td>0.12—0.28</td>
</tr>
<tr>
<td>AML (5)</td>
<td>0.11—0.20</td>
</tr>
<tr>
<td>ALL (3)</td>
<td>0.08—0.14</td>
</tr>
</tbody>
</table>

Phosphorylation of uracil arabinoside (ara-U) by leukocyte preparations. Cells are incubated for 15 minutes at 37°C in a medium containing 0.05 mM ara-U-3H. The free drug was washed from the cells, and the intracellular level of ara-U nucleotides was measured. CLL, chronic lymphocytic leukemia; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia.

*Numbers in parentheses refer to the number of separate cell samples obtained from different patients with leukemia or normal donors. The range of data obtained is shown.

The data presented on phosphorylation of ara-U are important since rapid deamination of ara-C to ara-U was found in some leukemic cell preparations (T. C. Hall, unpublished data; measurements carried out as described in Ref. 2). Since the capacity for ara-U phosphorylation in cell preparations tested was relatively low, the consequence of extensive ara-C deamination would be a lowering of the total amount of labeled nucleotides formed from labeled ara-C. The extent of ara-C deamination varied widely among the different cell preparations examined. Preliminary examination of the data has shown no correlations between capacities for ara-C deamination and ara-C phosphorylation. At present, it is not known to what extent treatment of leukocyte preparations with inhibitors of ara-C deamination would increase the extent of ara-C phosphorylation.

It is noteworthy that normal small lymphocytes and chronic lymphocytic leukemic cells showed a uniformly high capacity for ara-C phosphorylation. This finding suggests a trial of ara-C in chronic lymphocytic leukemia and for possible immunosuppressive action in man. Mature granulocytes and the immature granulocyte forms represented by marrow precursors and by the myelocytes and promyelocytes of chronic myelogenous leukemia showed a lower capacity for ara-C phosphorylation. An extremely wide range was found when the capacity of acute leukemia blast cells was tested for ara-C phosphorylation. This phenomenon might explain the varying ability of ara-C to produce remissions in acute leukemias.

The ara-C-resistant acute leukemia cells showed a markedly low capacity for phosphorylation of the drug; cells from patients later shown to have ara-C-sensitive leukemias demonstrated rapid conversion of the drug to nucleotides. These findings suggest that studies of ara-C phosphorylation by leukemic cells in vitro might be useful in predicting subsequent responses to the drug.

REFERENCES


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