The Effects of Arabinosylcytosine on Cultured Human Lymphoma Cells

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SUMMARY

Asynchronous human lymphoma cells incubated with various concentrations of 1β-D-arabinofuranosylcytosine (ara-C) for 1 hr did not exhibit loss of viability. A similar situation existed when ara-C was presented for 1 hr to synchronized cells at various points of the cell cycle. However, when treatment was extended for 48 hr in asynchronous populations, a decrease in survivors to about 2% was elicited. Synchronized S-phase cells exposed to ara-C for 14 hr also showed a decrease in survival to 4%. Incubation with ara-C inhibited triated thymidine uptake irreversibly for at least 8 hr after the drug was removed from the external environment. The fact that the lymphoma cells possess low levels of ara-C kinase may explain the ineffectiveness of the drug with short exposures. After 24 hr incubation, about 30% of the total intracellular ara-C had been incorporated into nucleic acids.

INTRODUCTION

ara-C² is an antitumor metabolite that can inhibit the reproduction of different kinds of organisms varying from DNA viruses (18, 37) to mammalian cells (12). It has therefore been used as an effective chemotherapeutic agent in both animal and human neoplasias (30). The antitumor activity is ascribed to its effect of inhibiting DNA synthesis after being phosphorylated to ara-CTP (6, 22). This inhibition may result by a direct effect on DNA polymerase (14, 27) with ensuing lethality by unbalanced growth (1, 25). Cell killing effects may be suppressed by concomitant incubation with deoxyctydine, 100 times the concentration of ara-C (40), although rescue of treated cells can be achieved only with very high doses of deoxyctydine and then only in cells not in S phase. It has been shown that certain cells need only to be incubated with the drug for very short periods and cell lethality will still occur even though the drug is removed from the external environment (2, 7). The antitumor activity of the drug has generated a variety of clinical protocols for the chemotherapeutic management of human neoplasias of lymphoid origin (30).

We are now engaged in the investigation of the effects of chemotherapeutic drugs at the cellular level. The effect of ara-C on an established line of human lymphoma cells constitutes this report.

MATERIALS AND METHODS

Cell Line. T1 cells, of a human lymphoma cell line (35), produce a tumor-specific antigen common to lymphomas (36). The cells are maintained in Ham's F-10 medium supplemented by 20% fetal calf serum, glutamine, vitamins, and antibiotics. Methods for the serial propagation have been previously reported (10). The kinetic values are: doubling time, 52 hr; generation time, 27 hr; G1, 3.5 hr; S, 13.5 hr; G2, 10 hr; MI, 1.6. Although the plating efficiency of T1 cells may range up to 60% (9), in the present series of experiments it varied from 20 to 40%.

Drug. ara-C (Cytosar, Upjohn Co., Kalamazoo, Mich.) was obtained in freeze-dried form containing 100 mg of drug per vial. The drug was reconstituted in 0.9% NaCl solution immediately before use, and appropriate dilutions were prepared in fresh medium. The pH always varied from 7.2 to 7.4.

Dose-Response Survival of Asynchronous Populations. Asynchronous T1 cells were dispensed into 60-mm Petri dishes (10⁶ cells/dish) and incubated for about 48 hr. The medium was discarded and the cells were exposed to increasing concentrations of ara-C for exactly 1, 24, or 48 hr at 37° in a 5% CO₂ humid atmosphere. The drug was decanted and the cells were washed twice with fresh medium. They were then harvested, counted in an electronic particle counter (Coulter Model F counter, Coulter Electronics, Hialeah, Fla.), and replated so that 50 to 100 colonies would appear after 21 days of incubation. The colonies were briefly rinsed with 0.9% NaCl solution, stained with 0.5% crystal violet in 95% ethanol, and examined under a stereomicroscope. A cell was considered viable if it gave origin to a colony of more than 50 cells. To mark the cells that were in S during the drug treatment we added TdR³H (1 μCi/ml; specific activity, 3.0 Ci/m mole) to replicate cultures and left it for the duration of the drug exposure. These cells were harvested, hypotonized with 1% sodium citrate, fixed with Carnoy's solution, and processed for radioautography by the liquid emulsion technique (Ilford K3). The slides were developed after 1 week, and 1000 cells were scored as labeled or unlabeled.

Single Dose as a Function of Duration of Treatment. The effects of a single concentration of ara-C, 500 μg/ml, as a
function of duration of treatment were investigated by dispensing the drug to several dishes at the time designated 0 hr and harvesting the cells of these replicates at regular intervals thereafter. The cells were plated for survival in the manner described in the previous section. Controls were selected at each test point.

Synchronization of T1 Cells. To obtain large numbers of cells synchronized at the beginning of the S period, we used a single treatment with 3 mM TdR. The degree of synchrony was monitored by 30-min pulse labeling with 1 μCi of TdR-3H per ml to determine the percentage of cells in S phase and by scoring the MI. At the end of the synchrony procedure, approximately 90% of the population was in early S phase and progressed into G2 and M synchronously. Cells in G1 phase were obtained by the use of a single treatment with 3 mM TdR of 24-hr duration followed by mitotic selection at the time calculated for the arrival of the cells at the end of G2 phase. The resulting MI ranged from 75 to 90% in different experiments. The mitotic cells were incubated at 37°, and the MI rapidly fell to zero, indicating that the cells had progressed into G1 phase.

Single Dose-Age Response. Age-dependent response to a single dose of ara-C (500 μg/ml) was studied by synchronizing the cells with the above-described methods and incubating them with the drug for 1 hr at various times throughout the length of the cell cycle. Synchrony was monitored by the MI and by pulse labeling additional dishes with TdR-3H for 30 min with subsequent radioautography. Controls were obtained from synchronized cells at each test point.

Prolonged Treatment of S-Phase Cells. Increasing concentrations of ara-C (1 to 100 μg/ml) were dispensed to TdR-synchronized S-phase cells immediately after the blocking agent was removed. The treatment continued for 14 hr, approximately the length of S. The treated cells and the controls (synchronized cells that did not receive the drug) were harvested, plated, and incubated in the usual manner.

Blocking Effects of ara-C. Asynchronous, logarithmically growing T1 cells received increasing concentrations of the drug (10, 50, 100, and 1000 μg/ml) for 1, 24, or 48 hr. After the prescribed incubation time the treatment medium was discarded, the cells were washed twice with fresh medium, and the cells were reincubated with fresh medium. Pulse labeling with TdR-3H was carried out in replicate cultures for each dose point at 0 hr and every 2 hr thereafter for 8 hr.

Radiochemicals. Generally triated ara-C (specific activity, 19 Ci/mmmole) and tetrahydouridine (NSC 112907) were supplied by the Drug Development Branch of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md. The radiochemical purity was greater than 97% as determined by chromatographic and radiochemical techniques. Other chemicals were commercially available.

Enzyme Assays. Asynchronous, logarithmically growing T1 cells were collected, washed 3 times with 0.9% NaCl, and sonically disrupted for 30 sec in 3 volumes of 0.25 M sucrose. The preparation was centrifuged for 1 hr at 100,000 X g, and the supernatant was recovered for the assay of ara-C deaminase (D) and ara-C kinase (K) activities. All of the above procedures were carried out at 4°, and the enzyme assay is described elsewhere (2).

Uptake of ara-C-3H. Ten million cells were incubated at 37° in a CO2 incubator with 10 ml of media containing ara-C, 100 μg/ml, and ara-C-3H, 2.5 μCi/ml. After the designated time of incubation, the cells were collected by centrifugation and washed 3 times each with 40 ml of ice-cold 0.9% NaCl. The cells were dispersed by a vortex mixer and denatured with 4% perhydrol acid. The supernatant (acid-soluble fraction) was recovered, neutralized, and applied on a Whatman No. 1 filter paper. The separation and identification of the radioactive peaks (ara-C, phosphorylated ara-C, and ara-U) have previously been described (20). The acid-insoluble materials were washed 2 times each with 40 ml of 4% perhydrol acid, 1 time with ethanol:ether (3:1), and then with ether. The final pellet was heated in a boiling water bath for 20 min. The hydrolysate was counted for tritium after a brief centrifugation. The analysis procedure was carried out at 4°. The controls were cells processed with drug added but without additional incubation.

RESULTS

Dose-Response Survival of Asynchronous T1 Cells. The survival of asynchronous T1 cells is shown in Chart 1. Treatment for 1 hr with doses as high as 1000 μg/ml did not elicit cellular lethality although 26% of the cells were in S phase. When the drug was left for 24 hr, a marked decrease in survival was observed with a plateau of 14% survivors beyond doses of 10 μg/ml. The labeling index indicated that 73% of the cells had passed through S during the 24-hr treatment period. The plateau of survival could be overcome, with a further increase in killing, with concentrations as high as 1000 μg/ml. At that dose point the survival decreased to 8.5%.

![Chart 1](https://example.com/chart1.png)

Chart 1. The effect of increasing concentrations of ara-C on T1 cells (asynchronous). Points, average value of 4 experiments with 3 replicates each.
Leaving the drug for 48 hr produced a substantially larger kill to a plateau of about 2% survivors reached with doses higher than 10 \( \mu g/ml \). During that period 83% of the cells had passed through S phase as indicated by the labeling index. Again a dose of 1000 \( \mu g/ml \) further decreased survival to 0.7%.

**Survival as a Function of Duration of Treatment.** The survival of \( T_1 \) cells treated with a single dose of 500 \( \mu g/ml \) decreased steadily as a function of the duration of exposure to the drug. Four hr after incubation, the survival had already decreased to 80% and finally, after 24 hr, the survival was in order of 17% (Chart 2).

**Single Dose-Stage Sensitivity.** Synchronized \( T_1 \) cells exposed to a single dose of 500 \( \mu g/ml \) for 1 hr at various times during the entire cell cycle did not reveal decrease in survival. The only exception was a modest decreased to 85% survivors in mid-G2. Dose-response survival experiments with synchronized cells were therefore not carried out.

**Prolonged Treatment of S-Phase Cells.** Cells synchronized in S phase with 3 mM TdR were exposed to increasing concentrations of ara-C (1 to 100 \( \mu g/ml \)) immediately after the synchronizing agent was removed. At that time 88% of the cells were in S. The cells were exposed to the drug for 14 hr. The survival reached a plateau of 4% with doses higher than 25 \( \mu g/ml \) in a manner similar to that seen with asynchronous cells treated for 48 hr (Chart 3).

**Blocking in S with ara-C.** \( T_1 \) cells treated for 1 hr with ara-C, 10, 50, 100, or 1000 \( \mu g/ml \), did not reveal any significant block of DNA synthesis as evidenced by a labeling index similar to that of the control. Incubating the cells for 24 hr with the above concentrations of ara-C produced a marked decrease in TdR-\( ^3 \)H uptake with doses higher than 100 \( \mu g/ml \) (Table 1). Even those cells that were considered labeled probably had a decreased uptake ratio since the number of grains per nucleus ranged from 5 to 15, whereas the control cells revealed 30 to 60 grains per nucleus. The inhibition of TdR-\( ^3 \)H uptake was seemingly irreversible after the cells were placed in fresh medium, since the number of labeled cells did not increase during the 8-hr sampling period. When the cells were treated for 48 hr the inhibition of TdR-\( ^3 \)H was even more substantial (Table 2). Cells treated with 10 to 100 \( \mu g/ml \) demonstrated very few labeled cells initially and minimal increase during the 8-hr observation period following removal of ara-C. Cells treated with ara-C, 1000 \( \mu g/ml \), demonstrated an absolute block in TdR-\( ^3 \)H uptake; no labeled cells were seen during the 8 hr following reincubation in fresh medium.

**Enzyme Levels.** The activity of ara-C kinase in \( T_1 \) cells was of 28 nmoles per g cells per hr and that of ara-C deaminase was of 35 nmoles per g cells per hr, giving a K/D ratio of 80.

**Uptake of ara-C-\( ^3 \)H.** Cells exposed to ara-C-\( ^3 \)H for 1 hr incorporated a substantial amount of radioactive drug. Almost the total amount of the drug was present in the acid-soluble fraction; some incorporation into nucleic acids was also detected (Table 3). The drug had been extensively

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment Duration (hr)</th>
<th>0 hr</th>
<th>2 hr</th>
<th>4 hr</th>
<th>6 hr</th>
<th>8 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

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**Chart 2.** The effect of ara-C on \( T_1 \) cells (asynchronous) as a function of duration of treatment. Points, average values of 3 experiments with 3 replicates each.

**Chart 3.** The effect of increasing concentrations of ara-C on synchronized S-phase cells. Points, average values of 3 experiments with 3 replicates each.
phosphorylated into ara-C nucleotides (61%), while a modest amount (12%) had been degraded to ara-U. In cells exposed for 1 hr to ara-C and reincubated in fresh medium for an additional 23 hr, all of the drug was converted to ara-C nucleotides and a considerable amount of these nucleotides had been incorporated into nucleic acids. T1 cells incubated with ara-C for 24 hr incorporated more than 3 times the amount of the drug that was observed in 1 hr treatment. However, the relative distribution of ara-C, ara-U, and ara-C nucleotides was similar in both treatments. Nevertheless, the amount of ara-C incorporated into nucleic acids was about 30 times more.

DISCUSSION

The mechanism of ara-C has been the subject of many conflicting reports. It was originally thought that the drug inhibited the enzyme ribonucleotide reductase (6), thus preventing the synthesis of deoxycytidine; the absence of this nucleotide would in turn inhibit the synthesis of DNA. Subsequently, it was shown that such was not the mechanism of action of the drug (32) and that ara-C actually inhibits DNA polymerase directly (14). However, it has also been indicated that inhibition of the polymerase occurs only when DNA synthesis takes place in the presence of a normal DNA template. DNA polymerase inhibition does not occur when synthetic polynucleotides are utilized as a template in cell-free systems (13). Other investigators have proposed that ara-C may prevent DNA synthesis and kill cells by incorporation into DNA chains (7, 33) or as a chain terminator at the 3'-hydroxyl terminal (31). ara-C induces chromosome breaks in treated cells (23) and does so more effectively in G2 cells (3). However, the general consensus is that cell lethality results from inhibition of DNA synthesis which induces a state of unbalanced growth (25). It has been indicated that, in order to obtain cell lethality, treatment with low doses of ara-C should be extended for at least a period equal to the length of S (21, 26). Suppression of cellular growth is achieved with 10^-6 M ara-C in continuous treatment. Nevertheless, cell lethality does not occur if the drug is left in contact with the cells for only 1 hr unless a concentration 100 times as high is utilized (40). However, we have shown that, in human melanoma cells sensitive to ara-C, cell killing is obtained with relatively low doses (10 Ìg/ml) after 1 hr treatment (2). Several investigators have shown that the inhibition of DNA synthesis may be reversible in some mammalian cells (28, 29) and that a state of even partial synchrony can be achieved in cells treated with ara-C (24). However, in some cell lines the cytotoxic effects of ara-C on S-phase cells are apparently irreversible (40), and therefore this drug may not be a useful synchronizing agent. In order to exert its activity ara-C must be phosphorylated to ara-CTP, a reaction mediated by deoxycytidine kinase (5, 22). The drug is also rapidly deaminated to ara-U by deoxycytidine deaminase (4, 8). Resistance of certain tumors to the effects of ara-C has been determined to be due to either high levels of deaminase (35) or low levels of kinase (17).

In our studies, asynchronous lymphoma cells did not exhibit loss of viability when treated with ara-C doses as high as 1000 Ìg/ml for 1 hr. However, exposure to ara-C for 24 or 48 hr evidenced greater lethality (Chart 1), and a plateau of survival could be reached with doses as low as 10 Ìg/ml in both cases. Cells exposed to ara-C for 48 hr had a decrease in survival to about 2%, while the number of cells that had passed through S was 83%. This suggests that ara-C may kill cells in stages of the cell cycle other than S. Treating the cells with a single dose of 500 Ìg/ml for long periods yielded a modest increase in killing effects. There were no fluctuations in survival along the cell cycle when ara-C, 500 Ìg/ml, was presented to the cells for 1 hr. However, when the drug was presented to the synchronized S-phase cells for the duration of S (Chart 3), a decrease in survival to 4% was obtained. This suggests that there is a minimum exposure time, even in the presumed sensitive stage, during which the drug must be in contact with the cells in order to obtain significant killing effects.

Table 2
Percentage of labeled cells after treatment with ara-C for 48 hr

<table>
<thead>
<tr>
<th>Dose of ara-C (Ìg/ml)</th>
<th>0 hr</th>
<th>2 hr</th>
<th>4 hr</th>
<th>6 hr</th>
<th>8 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>26</td>
<td>29</td>
<td>26</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
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<td>0</td>
<td>2</td>
<td>8</td>
<td>16</td>
<td>15</td>
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<tr>
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<td>16</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3
Uptake of ara-C in lymphoma cells in culture

<table>
<thead>
<tr>
<th>Acid-soluble fraction</th>
<th>ara-C</th>
<th>ara-U</th>
<th>ara-C nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of incubation with ara-C, 100 Ìg/ml (hr)</td>
<td>No. of studies</td>
<td>Total Ìg</td>
<td>% of total</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.47</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>0.10</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>4</td>
<td>1.7</td>
<td>25</td>
</tr>
</tbody>
</table>

a The drug was washed off and reincubated in fresh medium for 23 hr.
Therefore, in certain cells the product concentration times time (CX T) (19) can be manipulated only to a certain extent since it is obvious that, regardless of the dose used, a minimum treatment interval is required to produce cell lethality. When T<sub>1</sub> cells were exposed to ara-C for 1 hr, no significant block in DNA synthesis was observed. Treatment for 24 hr produced a marked decrease of DNA synthesis (under the assumption that inhibition of TdR<sup>3</sup>H uptake is synonymous with lack of DNA synthesis). An exposure of 48 hr produced an even greater effect. This inhibition was irreversible at least during the 8 hr of reincubation in fresh medium since no increment of TdR<sup>3</sup>H uptake was observed.

One plausible explanation for the limited effects of ara-C on T<sub>1</sub> cells is that these cells contain very low levels of ara-C kinase, which is the rate-limiting factor in the conversion to the effector molecule, ara-CTP. For generation of DNA inhibition and cell lethality, a critical concentration of ara-CTP is probably necessary. This concentration may be achieved if the rate of phosphorylation is higher than the rate of degradation to ara-U. T<sub>1</sub> cells possess low levels of both kinase and deaminase enzymes. Therefore, although the rate of conversion to ara-CTP may be minimal, deamination to ara-U also occurs and a sufficient number of ara-CTP molecules may accumulate in time. In T<sub>1</sub> cells, although the kinase activity was low, the major part of ara-C was rapidly converted into ara-CTP (over 60%) during 1 hr (Table 3). After 24 hr incubation with ara-C, 3 times as much ara-CTP was present in the acid-soluble fraction and 30 times as much had been incorporated into nucleic acids. A considerable amount of ara-CTP was incorporated into nucleic acids. Incorporation of ara-CTP into DNA and RNA has been observed in several mammalian cells (5, 8, 32), and it has been proposed that this incorporation results in the ultimate death of the cell (7). If ara-C does indeed inhibit DNA polymerase, it is difficult to understand how the ara-C can be introduced into the DNA chain by the same enzyme it purportedly inhibits. Several possibilities can occur. (a) Inhibition of DNA polymerase is only relative and dose dependent. T<sub>1</sub> cells exposed to doses as high as 1000 μg/ml for 24 hr still had a small percentage of cells incorporating TdR<sup>3</sup>H. At low doses of ara-C, DNA synthesis occurred in a percentage of cells similar to that in the controls, although possibly at a lower rate as suggested by the paucity of grains. (b) Inhibition of DNA polymerase depends on the type of polymerase. There are more than 1 kind of DNA polymerases (38) and only some may be susceptible to the effects of ara-C. Different cell lines may have different proportions of these enzymes. A similar situation occurs in certain cell lines refractory to the effects of amethopterin. These cells contain different proportions of folic acid reductases only some of which may be inhibited by the drug (16). (c) The major locus of the effect of ara-C may be on the DNA template not by incorporation into the chain but perhaps by forming complexes with the DNA. This effect may inhibit the replication of DNA without affecting DNA polymerase directly. (d) Finally, cell lethality may be the result of 2 consecutive mechanisms. ara-C may inhibit DNA polymerase in an irreversible fashion to a certain extent, and many cells die by the mechanism of unbalanced growth. In those cells where the drug does not absolutely inhibit the enzyme some ara-C is incorporated into nucleic acids, where it may cause strand breaks or terminal blocking of chain elongation. Our studies do not resolve these questions. However, they indicate the following clinical implications. (a) ara-C should be effective in neoplastic cells only if they have sufficient levels of ara-C kinase to permit intracellular accumulation of a lethal concentration of ara-CTP. (b) Low levels of ara-C kinase could be overcome by continuous therapy either in drip infusion or by a single injection of a slow-releasing form of ara-C. However, this would not be desirable since normal rapidly proliferating cells, which contain considerable levels of kinase, will be affected more severely by the continuous treatment. (c) Even in synchronized S-phase cells, therapy with ara-C should be extended for a minimum of time at least equal to the length of S to obtain a significant degree of kill.

REFERENCES


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