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SUMMARY

The basis of sensitivity and resistance of various Chinese hamster cell lines to the synthetic nucleoside, cytosine arabinoside, was studied. This agent was found to have an inhibitory effect on both DNA and protein synthesis. There was a differential but inhibitory effect on the synthesis of histones and other proteins. Survival studies showed an increasing probability of death with dose and time of exposure, in agreement with the hypothesis that probable errors in protein synthesis or regulation result in the observed unbalanced growth and death of cells sensitive to cytosine arabinoside.

Studies of hybrids constructed from isolated resistant and sensitive cell lines showed that resistance is a recessive phenotype, and a case of interallelic complementation was found. A nucleoside diphosphate reductase enzyme(s) appears to be involved in the resistance to cytosine arabinoside of the resistant Chinese hamster cells investigated in this study.

INTRODUCTION

 ara-C is a synthetic nucleoside similar in structure to cytidine and CdR, with the C-2' hydroxyl cis to the glycosyl linkage. The triphosphate of ara-C has been found to be an effective inhibitor of DNA synthesis in mammalian cells, as shown by competitive inhibition with dCTP of DNA polymerase (11). It has been used in the treatment of leukemia because of its cytotoxicity to cells in the S phase. CdR reverses the inhibition of DNA synthesis by ara-CTP (9). Although ara-C is incorporated into DNA (24), its cytotoxic effect is not correlated with this incorporation (13), nor is the effect due to inhibition of DNA synthesis per se, as it can be reversed by removal of the inhibitor (12).

Mouse leukemic cells in vitro that are resistant to ara-C have been isolated and studied (1, 10, 23). The basis of resistance can be any mutation that prevents the conversion of ara-C to ara-CTP (the cytotoxic form of ara-C), that increases the production of dCTP relative to that of ara-CTP, or that increases conversion of ara-C to a nontoxic form. This means that resistance is probably due to either an altered permease, cytidine kinase, CMP kinase, ribonucleoside diphosphatase, reductase, dCDP kinase, DNA polymerase, nucleoside deaminase, or gene products involved in feedback regulation of these enzymes.

This study investigates the basis of sensitivity and resistance to ara-C in Chinese hamster cell lines grown in vitro. The procedures used involve (a) the determination of cell survival as measured by plating efficiency, (b) the measurement of DNA and protein synthesis in cells under various conditions, and (c) the use of cell hybrids to study the phenotypic expression of resistance.

MATERIALS AND METHODS

Cell Lines and Medium. The aneuploid cell line V79-4 was originally derived from the lung tissue of a male Chinese hamster. From this parental line, several sublines were isolated: 366-18, which is resistant to 8-azaguanine (8); V79-426, which exhibits normal growth at 37 and 41°; and 462-10, which is resistant to BrdUdR (7). The first 2 cell lines each have 20 modal chromosomes, and the BrdUdR-resistant line has 18. The medium for all studies was F12 (14) without antibiotics.

Incorporation of Radioactive Isotopes. Cells were inoculated into 60-mm Petri plates containing 5 ml of medium and then were pulse labeled for 55 or 60 min with radioactive isotopes. Each plate was inoculated with 0.2 ml of one of the following stock solutions of radioactively labeled compounds: thymidine-3H, H, 10 μCi/ml; thymidine-3H, 10 μCi/ml; thymidine-3H, 5μCi/ml, plus leucine-3H, 5μCi/ml; or thymidine-3H, 5μCi/ml, plus arginine-14C, 5 μCi/ml. The original specific activities were 18.5 Ci/mmol, 50 Ci/mmol, 150 μCi/mmol, and 150 μCi/mmol for thymidine-3H, leucine-3H, leucine-14C, and arginine-14C, respectively. Usually, cells in 3 plates were collected, and the radioactivity was counted and averaged for each data point. Because the undialyzed fetal bovine serum contains the corresponding unlabeled compounds in unknown amounts, the average specific activities after dilution were not estimated. The method used to extract the
Diana B. Smith and Ernest H. Y. Chu

acid-insoluble fraction from cells was as described by Regan and Chu (21). Radioactivity was counted on a Packard liquid scintillation spectrometer.

Cell Hybridization and Chromosome Preparation. For each pairwise combination, 10⁵ cells of each parental line were added to a test tube. The tube was then centrifuged, the medium was decanted, and 0.2 ml of β-propiolactone-inactivated Sendai virus (400 to 1000 hemagglutination units) was added. The tube was left in ice for 15 to 20 min and then was incubated at 37° for 30 min. Five ml of preconditioned F12 containing 10⁻⁷ M thymidine, 10⁻⁵ M hypoxanthine, 3.2 X 10⁻⁶ M aminopterin, and 10⁻⁴ M glycine were added to each tube. One ml of this cell suspension was put into each of four 60-mm dishes containing the same selective medium. This medium was changed every 3 days. Ten to 14 days later, the surviving presumptive hybrid clones were isolated. A sample of each hybrid cell line was exposed to Colecemid, 0.03 µg/ml, for 3 to 4 hr, trypsinized, and suspended in warm (37°) hypotonic solution (0.7% sodium citrate) for 15 min. The cells were then spun down and fixed in a 3:1 mixture of absolute ethanol and glacial acetic acid. Several drops of cell suspension were placed on a wet microscope slide and air dried on a slide warmer. The slide was stained in carbol fuchsin for 15 to 20 min (5), and the number of chromosomes per cell was counted.

Inhibitors and Virus. ara-C hydrochloride was a gift of the Upjohn Company, Kalamazoo, Mich. A 3.6 X 10⁻⁷ M concentration of ara-C is equivalent to 0.1 µg/ml. A sterile stock solution of 1000 µg/ml in medium without serum was made, and 3.2-ml aliquots were frozen in small vials. Appropriate dilutions were made from these stock solutions for each experiment. Hydroxyurea was prepared as needed from a sterile stock solution of 1000 µg/ml (1.32 X 10⁻² M). Puromycin was prepared as needed from a sterile stock solution of 1000 µg/ml (1.84 X 10⁻³ M). β-Propiolactone-inactivated Sendai virus was provided by Dr. R. Tennant.

RESULTS

Cell Survival after Exposure to ara-C-supplemented Media. Table 1 shows the percentage of survival after a 24-hr exposure of V79-4 cells to various concentrations of ara-C. The average generation time of this cell line is approximately 12 hr. When exposed to high concentrations of ara-C for 24 hr, however, the majority of the cells failed to divide, except for a few that divided once. These probably were already in G₂ at the time of inoculation. The probability of cell survival and colony formation decreased with ara-C concentration.

Table 1

<table>
<thead>
<tr>
<th>Concentration of ara-C (µg/ml)</th>
<th>No. of cells</th>
<th>No. of plates</th>
<th>Relative survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>6</td>
<td>100.00</td>
</tr>
<tr>
<td>0.24</td>
<td>100</td>
<td>3</td>
<td>79.37</td>
</tr>
<tr>
<td>1.22</td>
<td>100</td>
<td>3</td>
<td>16.18</td>
</tr>
<tr>
<td>2.43</td>
<td>100</td>
<td>3</td>
<td>2.18</td>
</tr>
</tbody>
</table>

Table 2 shows the survival of 3 different hamster cell lines in the continuous presence of different levels of ara-C. Many of the cells at these low concentrations of ara-C went through several divisions, forming abortive colonies before dying. Cells in the presence of ara-C appeared enlarged and grew more scattered in colonies than under normal conditions. There were also quantitative differences among these cell lines in their growth response to the drug.

Chart 1 shows the survival of line 462-10 cells for various times in the presence of 0.1 µg of ara-C per ml in the medium. After treatment for selected periods, the cells were incubated in fresh medium until the 9th day, when they were fixed and stained and the plating efficiency was determined. For this particular cell line, the critical period of cell killing appears to be between 1 and 2 days after exposure to ara-C.

A resistant cell clone designated CA7 was isolated from cell line 462-10 after having been continuously exposed to 0.1 µg of ara-C per ml. The growth response of the clonal isolate (compared with that of the parental cell line) was plotted in Chart 2. Similar patterns of resistance were observed in all other isolates from the 3 parental lines after continuous exposure to 0.1 µg of ara-C per ml. On the other hand, isolates among survivors after 24 hr of exposure to higher concentrations of ara-C (at approximately the same level of survival) consistently exhibited a lack of resistance to the drug.

Formation of Cell Hybrids and Resistance of the Hybrids to ara-C-supplemented Medium. Because ara-C-resistant cell lines were isolated from cell lines that are resistant to either 8-azaguanine or BrdUdR, it was possible efficiently to select colonies of cell hybrids formed by fusion between the 2 different cell types. It has been shown (8) that the 8-azaguanine-resistant cell line lacks hypoxanthine-guanine phosphoribosyltransferase activity. The BrdUdR-resistant cell line fails to incorporate either thymidine-³H or BrdUdR-³H and thus probably lacks thymidine kinase activity (7). Both parental cell lines are known to be nonrevertible to the wild type (6, 7). Aminopterin is a folic acid antagonist that blocks dihydrofolate reductase and thereby disrupts the biosynthetic pathways of purines and thymidylate. This inhibition of purine and thymidylate synthesis can be circumvented by the addition of exogenous thymidine, hypoxanthine, and glycine to the medium (17), thus permitting selective growth of hybrids.
β-Propiolactone-inactivated Sendai virus was used to increase the frequency of cell fusion. The resulting presumptive hybrid clones were isolated and confirmed as hybrids by examination of chromosomes. Hybrids from combinations between cells derived from the 8-azaguanine- and BrdUdR-resistant cell lines used in this study had an average of 38 chromosomes (i.e., one parental line had 20 and the other had 18).

Fifteen different hybrids between ara-C-resistant cells, and between sensitive and resistant cells derived from the parental BrdUdR- and 8-azaguanine-resistant cell lines, were tested for their resistance to 0.1 μg of ara-C-supplemented medium per ml. Hybrids between sensitive and resistant cell lines were phenotypically sensitive to ara-C, whereas hybrid combinations between all but 1 pair of ara-C-resistant cell lines (CA7 × CA18) were resistant to the drug. The 2 different types of growth response of cell hybrids in ara-C medium are illustrated in Table 3.

**Effect of Hydroxyurea on DNA Synthesis as measured by Incorporation of Thymidine-$^3$H.** Hydroxyurea is a known inhibitor of ribonucleoside diphosphate reductase in *Escherichia coli* (16). In order to test the hypothesis that resistance to ara-C may be due to a mutation directly or indirectly affecting this enzyme, we compared ara-C-resistant clone CA19 with its parental cell line (386-18) for their response to a 2-hr treatment with various concentrations of hydroxyurea. After 1 hr of treatment, the cells were labeled with thymidine-$^3$H for the next hr in the continued presence of the inhibitor. Chart 3 shows the relative incorporation of thymidine-$^3$H into the acid-insoluble fraction of cells in the presence of hydroxyurea. This experiment was repeated 3 times, and it consistently showed that CA19 was more sensitive to hydroxyurea than its parental cell line, 386-18.

When various hybrids were tested in the same manner for response to hydroxyurea, 10 μg/ml, 2 general levels of

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**Table 3**

<table>
<thead>
<tr>
<th>Parental cell line</th>
<th>462-10 (S)</th>
<th>CA7 (R)</th>
<th>CA9 (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>386-18 (S)</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>CA18 (R)</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>CA19 (R)</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

* S, sensitivity to ara-C; R, resistance to ara-C.
Diana B. Smith and Ernest H. Y. Chu

Chart 3. DNA synthesis as measured by relative incorporation of thymidine-3H (1-hr pulse) into the acid-insoluble fraction after a 1-hr exposure of cells to various concentrations of hydroxyurea. x, cell line 386-10; o, cell line CA19 (derived from cell line 386-18 after continuous exposure to ara-C, 0.1 μg/ml. Values are relative to the control. CONCN., concentration.

Resistance were found, 1 near the parental line and 1 much higher; these varied from one hybrid combination to another. Table 4 gives some of these results, in percentage of control, for those hybrids also mentioned in Table 3.

Effect of CdR on Resistance to ara-C as Measured by DNA Synthesis and Plating Efficiency. CdR reverses the inhibition of DNA synthesis by ara-C (9). Chart 4 shows this effect of CdR on cell lines CA19 and 386-18. The amount of ara-C was 0.1 μg/ml and that of CdR was 10 μg/ml. CdR completely overcame the inhibitory effect of ara-C on the sensitive cells, the result being a level of cell survival comparable to that obtained in F12 alone or in F12 plus CdR. By contrast, the resistant cell line was less affected by CdR supplemented to ara-C medium.

Effect of Puromycin on DNA and Protein Synthesis. The effect of 20 μg puromycin per ml on DNA and protein synthesis was studied in cell line V79-426. In different experiments, protein synthesis was measured by the incorporation of either radioactively labeled arginine or leucine into cells in the presence or absence of puromycin. In both experiments puromycin was added at time zero. One hr before each pulse labeling, the medium was changed to one not containing the amino acid used for labeling protein synthesis but still containing puromycin. The time of pulse labeling was 55 min. The results are expressed as percentage of control at the same hr.

Chart 5A shows the typical results of the 1st type of experiment, in which arginine-14C and thymidine-3H were used for labeling protein and DNA, respectively. Chart 5B shows the results of an experiment with thymidine-3H and leucine-14C. In both cases the inhibition of DNA synthesis is nearly the same, but the inhibition of protein synthesis is strikingly different. Incorporation of leucine is much more inhibited than that of arginine.

Effect of ara-C on DNA and Protein Synthesis. The effect of

<table>
<thead>
<tr>
<th>Parental cell line (%)</th>
<th>462-10</th>
<th>CA7</th>
<th>CA9</th>
</tr>
</thead>
<tbody>
<tr>
<td>386-18α</td>
<td>21</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>CA18</td>
<td>Not done</td>
<td>72</td>
<td>15</td>
</tr>
<tr>
<td>CA19</td>
<td>18</td>
<td>Not done</td>
<td>85</td>
</tr>
</tbody>
</table>

α Had 21% incorporation.

Chart 4. A, DNA synthesis in an ara-C-resistant cell line CA19, as measured by thymidine-3H incorporation after 1-hr pulse labeling, in the presence of various additives in the medium: x, no additive; o, ara-C at 0.1 μg/ml; o, ara-C at 0.1 μg/ml plus CdR at 10 μg/ml; and ▲, CdR at 10 μg/ml. Values are relative to time-zero control incorporation. B, DNA synthesis in ara-C-sensitive cell line 386-18, as measured by thymidine-3H incorporation after 1-hr pulse labeling, in the presence of various additives in the medium. x, no additive; o, ara-C at 0.1 μg/ml; o, ara-C at 0.1 μg/ml plus CdR at 10 μg/ml; ▲, CdR at 10 μg/ml. Values are relative to time-zero control incorporation.
Resistance of Hamster Cells to ara-C

1. Resistance of Hamster Cells to ara-C supports the view that the cause of death is not simply inhibition of DNA synthesis. Furthermore, the distorted morphology of cells in abortive colonies suggests that unbalanced growth might have taken place in the presence of ara-C.

Graham and Whitmore (12) have stated that ara-C has little effect on RNA and protein synthesis. However, since ara-C is known to affect histone synthesis (3), which is correlated with DNA synthesis, it should affect the synthesis of both DNA and proteins. The effect of puromycin (27), as studied here by 2

2 different concentrations of ara-C (1 and 10 µg/ml) on DNA and protein synthesis in cell line V79-426 was studied by procedures similar to those used in the above experiments. The results are given in Chart 6, A and B. Although protein synthesis is affected in both instances and inhibition increases with concentration of ara-C, the magnitude of the inhibition is different. Chart 6C gives the results of labeling different plates with thymidine-3H and leucine-3H without removing leucine from the medium. In all 3 experiments, DNA and protein synthesis was less affected by 1 µg or ara-C per ml than by 10 µg/ml.

**DISCUSSION**

**Cause(s) of Cell Death in the Presence of ara-C.** ara-C has a lethal effect on Chinese hamster cells grown in culture, but this effect is partially reversible, depending on the dose and duration of exposure. Studies of cell survival as a function of increasing dose suggest that, at certain concentrations of the drug, changes in feedback regulation or other cell functions alter the chances of death. The fact that many cells can go through several divisions at very low doses before dying supports the view that the cause of death is not simply inhibition of DNA synthesis. Furthermore, the distorted morphology of cells in abortive colonies suggests that unbalanced growth might have taken place in the presence of ara-C.

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Note: The image contains charts and graphs that are not described in the text. The text describes experiments and observations related to the effects of ara-C on DNA and protein synthesis in cell line V79-426. The charts and graphs illustrate the results of these experiments.
different labeling methods (leucine$^{14}C$ versus arginine$^{14}C$), supports the evidence (28) that this drug has a differential effect on protein synthesis by way of ribosomes, in contrast with the nonribosomal synthesis of histones through aminoacyl-tRNA transferase (25). In mammalian cells, there is such a nonribosomal-acting transferase enzyme for arginine, but there is none for leucine. With the same labeling methods, it was further demonstrated that the synthesis of arginine-rich proteins was affected by dose of ara-C but to a different extent than was the synthesis of proteins containing leucine.

Inhibition of DNA and protein synthesis by ara-C was also shown to be dose dependent. The most likely cause of cell death seems to be errors in either the regulation or synthesis of S-phase proteins, because these errors cannot be reversed, once the cell leaves the S phase, at high doses of ara-C. This interpretation is supported by findings (2, 4) that chromosome breaks induced by ara-C in S cannot be reversed by subsequent (after S) addition of CdR. Recently, M. Y. Chu and G. A. Fischer (personal communication, 1972) showed that loss of cell viability is correlated with incorporation of ara-CTP into low-molecular-weight RNA.

**Mechanism of Cell Resistance to ara-C.** Of the possible mechanisms of resistance, alterations in CdR kinase (10, 23), ribonucleoside diphosphate reductase (25), and even DNA polymerase (1) have already been suggested or implicated by others as the basis of resistance in cell lines studied. It is also possible that an increased activity of nucleoside deaminase in the resistant cell may detoxify ara-C that enters the cell.

In this investigation, the difference in survival of related hamster cell lines in the presence of ara-C suggests that some genetic differences between these cell lines may have a partial effect on the degree of resistance. In particular, thymidine resistance has been associated with increased resistance to ara-C by some investigators (1). In agreement with these observations, the BrdUdR cell line used in the present studies, which is to incorporate thymidine$^3H$, has the highest resistance to ara-C at higher doses (0.1 µg/ml) as compared with the other cell lines studied. Morris and Fischer (19) suggest that a phosphorylated derivative of thymidine inhibits the conversion of CMP to dCMP.

At the level of ara-C (0.1 µg/ml) at which resistant clones were isolated, the data implicate only 1 enzyme for the major effect on resistance. If isolates were obtained at higher ara-C levels, spontaneously or after mutagen treatment, the primary basis of resistance might change to that of another altered enzyme. This is inferred from the ability of ara-C to inhibit DNA synthesis to some extent even in resistant cells. Inhibition in this case is a matter of degree, not an all-or-nothing phenomenon.

In *E. coli*, hydroxyurea is an irreversible allosteric inhibitor of ribonucleoside diphosphate reductase (16). This enzyme, in *E. coli*, is a dimer composed of 2 unequal subunits, one of which may contain the catalytic site and the other the allosteric site (21). In mammalian cells, the enzyme structure is unknown, and there appears to be a cytidine-specific soluble and insoluble form of this enzyme (15), as well as a general reductase. Whether hydroxyurea inhibits one or all in mammalian cells is not known. The fact that the ara-C-resistant hybrids show such a variability in response to hydroxyurea suggests the involvement of one or all of those with resistance to ara-C. Momparler et al. (18) suggest that, in an ara-C-resistant mutant line in mouse leukemic L5178Y cells with an increased pool size of dCTP, resistance may be explained by either "(1) an increased production of CDP reductase, (2) an increased level of CDP reductase activity due to an alteration of the concentrations of allosteric effectors of this enzyme, or (3) the response of CDP reductase to allosteric effectors due to changes in the conformation of the allosteric effector site of the enzyme."

Our results imply that changes may have occurred that affect either the conformation of the allosteric site of the enzyme or the concentration of allosteric effectors, as indicated by response of hybrids to hydroxyurea (Table 4).

If, in the present study, the resistance shown by most of the ara-C isolates was due to an alteration in the structure of a ribonucleoside diphosphate reductase, then resistance could be explained as a preferential production of dCTP, rather than ara-CTP, in the resistant cells. In a preliminary experiment on induction of ara-C-resistant mutations by ethyl methanesulfonate, the frequency of mutation was affected by the initial size of cell inoculum. It is possible that the sensitive wild-type cells, because of metabolic cooperation (26), had the ability to kill adjacent resistant mutant cells by the transfer of a derivative of ara-C.

In cell fusion experiments, resistance to ara-C behaves phenotypically like a recessive character in cell hybrids. The lack of complementation between paired resistant parental cells (e.g., CA9 × CA18, CA7 × CA19, and CA9 × CA19 (Table 3]) implies that they are defective in the same gene product. On the other hand, the sensitivity of a particular hybrid combination (CA7 × CA18) to ara-C may be explained by the formation, by complementation, of a normal functioning enzyme that causes death of the hybrid in an ara-C-supplemented medium. Since different mutants apparently reflect different changes in the same polypeptide chain, the formation of a functional enzyme in this particular cell hybrid may be taken as evidence for interallelic complementation in these mammalian cells. This conclusion is similar to that of Nadler et al. (20), who demonstrated complementation for the enzyme galactose 1-phosphate uridylyltransferase in cell hybrids from cell lines derived from different patients with galactosemia.

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**REFERENCES**


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