THE ACTION OF ARABINOSYLCYTOSINE ON SYNCHRONOUSLY GROWING POPULATIONS OF MAMMALIAN CELLS

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Arabinosylcytosine (1-β-D-arabinofuranosylcytosine, ara-C) has been reported to inhibit the reproduction of several different kinds of organisms ranging in complexity from DNA viruses to mammalian cells (Smith, 1965). A prominent finding, apparently related to the drug's effect on replication, has been the marked inhibition of DNA synthesis in organisms treated with ara-C (Chu and Fischer, 1962). Deoxycytidine will reverse the cytotoxic effects of the drug under certain circumstances (Silagi, 1965). These observations suggest that a disruption of DNA synthesis caused by ara-C is responsible for its cytocidal effect. Since DNA synthesis is confined, in mammalian cells, to a specific portion of the cell cycle, treatment of cells with this drug during their S phase of growth should produce a maximum amount of cell kill.

The action of ara-C on cells and the reversal and prevention of its cytotoxic effect during different stages of the cell cycle are reported here. Deoxycytidine prevents the cytotoxicity caused by the drug in cells in all stages of the cell cycle, but reverses the cytotoxicity only in G1 cells. The cytotoxicity caused by ara-C in S phase cells could not be reversed by deoxycytidine.

MATERIALS AND METHODS

The cell line used in these studies was Don C. It was maintained in Eagle's minimal essential medium, Earle's salts, (GIBCO) supplemented with

nonessential amino acids and fetal bovine serum (GIBCO). Cell cultures carried as stock lines were subcultured daily.

The method described by Stubblefield and Klevecz (1967) was used to obtain synchronously growing populations of cells. The parent population from which the synchronized population was to be selected was subcultured 17 hours before Colcemid (CIBA) was added at a final concentration of 0.06μg/ml. After a further incubation of 2.5 hours, the drug containing medium was poured off and replaced with a 0.1% trypsin (Worthington) solution. The flasks were shaken for twenty seconds, and the detached cells poured off and collected by centrifugation. The cells were diluted into culture medium and the time at which these cultures were returned to 37° C was considered to be the beginning of an experiment (t = 0). Points representative of the various stages were taken between t = 1.25 to 2.25 hours for G1; t = 3.25 to 4.25 hours for the first part of S (S_1); t = 6.25 to 7.25 hours for the latter part of S (S_2); and t = 8.75 to 9.75 hours for G2. Since synchrony degenerates towards the end of the initial cell cycle, there may be a mixed population of cells in G2.

Cell volume and cell number were determined by use of a Coulter electronic cell counter, Model B, with a size distribution plotter, Model H.

In all experiments with either logarithmic or synchronized cells the effect of one hour's treatment with ara-C on the ability of cells to multiply is reported as a percentage of the number of cells present, after two days incubation, in the non-ara-C treated control cultures, which is considered to be 100% outgrowth. By rescue is meant that cultures treated with ara-C are washed free of the drug before a second compound(s) is added to the culture medium. By protection is meant that cultures treated with ara-C are provided with a second compound(s) both during drug treatment and after the drug has been removed. The deoxynucleoside mixture used to rescue and protect ara-C treated cells consisted of deoxythymidine, deoxyadenosine, and deoxyguanosine each at 5×10^{-6} M, and deoxycytidine at 5×10^{-5} M.

Ara-C was obtained from Dr. J. H. Hunter of the Upjohn Chemical Company.

Deoxynucleosides were purchased from Schwarz BioResearch.

RESULTS

The effect of the continuous presence of ara-C on logarithmically growing cells was determined in order to find a drug level which would stop cellular multiplication (Chart 1). Inhibition of cell growth with respect to the ara-C concentration is approximately that reported for other cell lines (Chu and Fischer, 1962).

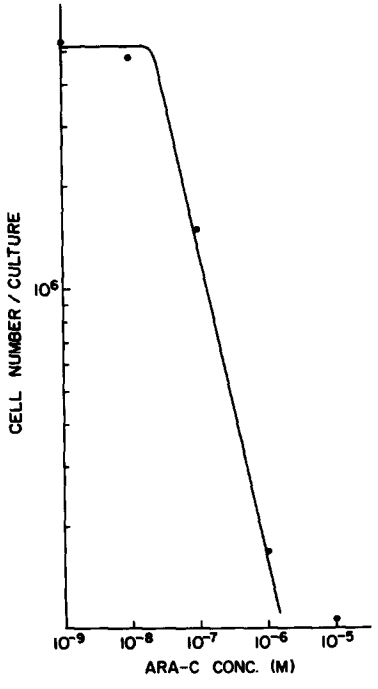

Chart 1. Survival of ara-C treated cells. Cells were inoculated at 1.9×10^5 cells per culture. Ara-C was added as indicated. After two days of incubation, the number of cells per culture was determined.

Since the length of incubation with the drug in experiments using synchronous populations of cells was limited to one hour in each of the various periods of the cell cycle, the effect of one hour's treatment with ara-C on non-synchronized cells was studied (Chart 2). At a final concentration of 1×10^{-5} M, ara-C has a significant effect on cellular multiplication, and the

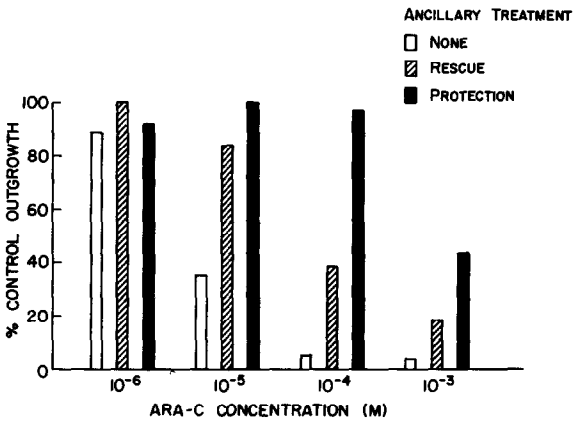

Chart 2. Effect of ancillary treatment on ara-C treated logarithmically growing cells. Cells were inoculated at 3.11 X 10^5 cells per culture three hours before ara-C was added at different final concentrations. The duration of drug treatment was one hour. Some cultures were rescued and others protected by a deoxynucleoside mixture.

drug suppresses all cell replication at a concentration of 1 X 10^{-4} M. The mixture of deoxynucleosides is effective in rescuing cells treated with ara-C at 1 X 10^{-5} M, but is only partially effective in rescuing cells treated at 1 X 10^{-4} M. Protection of the cells by the deoxynucleoside mixture is virtually complete with cells treated with ara-C at 1 X 10^{-4} M. Since ara-C at a final concentration of 1 X 10^{-4} M prevents cellular multiplication, but is only partially effective when deoxynucleosides are present after drug treatment, this drug level was studied with synchronous populations of cells.

Cells at all stages of the cell cycle treated with ara-C at a final concentration of 1 X 10^{-4} M are almost completely protected by the deoxynucleoside mixture, whereas cells which are not protected or rescued are completely inhibited in their growth (Chart 3). At this drug level, G1 treated cells are respons to rescue by deoxynucleosides; in contrast only a small fraction of S_1 treated cells respond. Rescue is progressively better with S_2 and G2 cells. Since the difference in response to rescue between cells treated in adjacent stages of the cell cycle is clear, the differential effect of deoxynucleosides on G1 and S_1 cells treated with ara-C was studied more closely.

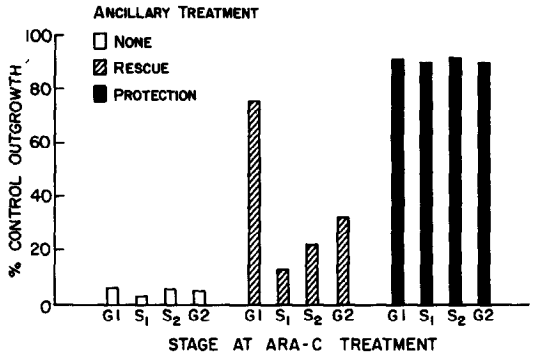

Chart 3. Effect of ancillary treatment on ara-C treated synchronized cells. Metaphase cells were inoculated at 2.5 X 10^5 cells per culture and incubated for one hour with ara-C at a final concentration of 1 X 10^{-4} M at representative points in the cell cycle. Some cultures were rescued and other cultures were protected by a deoxynucleoside mixture.

When different combinations of deoxynucleosides were tested for their ability to rescue G1 or protect S_1 cells treated with ara-C, only deoxynucleoside combinations containing deoxycytidine were able to rescue or protect drug treated cells. At a level of 1 X 10^{-4} M, deoxycytidine protects and rescues drug treated cells as well as the deoxynucleoside mixture.

The addition of deoxycytidine to G1 cells treated with ara-C is not effective in rescuing them; the drug had to be removed from the medium before rescue is accomplished. Rescued G1 cells subsequently grow logarithmically with the same doubling time as non-treated cells. With S_1 cells protected by deoxycytidine, the removal of the drug from the medium is not sufficient to allow the drug treated cells to resume their growth. In this circumstance deoxycytidine had to be present for two hours after the drug had been removed in order to achieve the same degree of protection provided to drug treated cells cultured continuously in the presence of deoxycytidine.

Ara-C treated cells do not divide after treatment, but steadily increase in volume (Table 1). Drug treated cultures synthesize as much protein as control cultures even though there are many more cells in the latter.

TABLE 1

PROTEIN SYNTHESIS BY ARABINOSYLCYTOSINE TREATED CELLS

Sample	Cell number X 10^6	mean cell volume, cubic microns	total protein per culture, mg	protein per 10^6 cells, μg
control	6.47	1250	1.26	195
G1	3.07	2536	1.22	397
S	3.12	2536	1.18	379

2.5 X 10^6 metaphase cells were inoculated per culture. These were incubated for one hour with ara-C (1 X 10^{-4} M) in G1 and S_1 . Twenty-one hours after the cultures were returned to 37° C, the cells were collected and the number of cells, cell volume, and cellular protein (method of Lowry) were determined.

When G1 cells are first treated with ara-C, rescue could be accomplished by deoxycytidine given as late as eight hours after drug treatment. The percentage of cells which grow out of the cultures rescued eight hours after drug treatment (72%) compares favorably with that of cultures rescued by deoxycytidine immediately after drug treatment (86%).

DISCUSSION

Although ara-C at a final concentration of 1 X 10^{-6} M suppresses the growth of logarithmically growing cells when present in the medium continuously, a drug concentration 100 times greater is necessary to achieve the same inhibition of growth when cells are treated with the drug for only one hour. At a drug concentration of 1 X 10^{-4} M, however, the death of some of the ara-C treated cells is prevented by the addition of deoxycytidine after drug treatment

When the inhibitory effect of ara-C is examined in synchronously growing cells, cells in all stages of the cell cycle are killed by one hour's treatment with the drug at a final concentration of 1 X 10^{-4} M. The lethal effects of the drug could be prevented, however, by the addition of deoxycytidine along

with the drug, or by giving G1 cells deoxycytidine after treatment with the drug. Cells which are synthesizing DNA on the other hand cannot be rescued by the addition of deoxycytidine after drug treatment.

Cells treated with ara-C do not divide, but continue to synthesize protein and enlarge in volume. This form of growth in G1, ara-C treated cells is reversible within the time period of the first cell cycle.

This data suggest that ara-C acts specifically and irreversibly to kill cells during the S phase of the cell cycle. Although unbalanced growth appears to take place in all cells treated with ara-C, there is a qualitative difference in the process in G1 and S drug treated cells. The observation of unalterable lethality in S cells implies that the biochemical lesion caused by ara-C is closely involved with the process of DNA replication.

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