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RETENTION OF 1-β-D-ARABINOFURANOSYLCYTOSINE *IN VITRO*— A SOURCE OF EXPERIMENTAL ERROR

MARK EDELSTEIN and FRED VALERIOTE

Section of Cancer Biology, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

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Abstract—Anomalous results of dose–survival curves from studies *in vitro* with the nucleoside analogue 1-β-D-arabinofuranosylcytosine (ara-C) led to a consideration of distribution of drug between cells and overlying culture media. The investigations presented here, using both a biological and a radioisotope technique, demonstrate that approximately 0.5 to 1 per cent of an initial dose of ara-C is released within 1 hr into the media from treated cells and the culture dish after an initial wash.

1-β-D-Arabinofuranosylcytosine (ara-C) is a cytidine analogue of both biological and clinical interest. Its importance in oncology results from its cytotoxicity to proliferating cells, especially those in the DNA synthetic phase of the cell cycle [1]. Its active triphosphate form, ara-CTP, is an inhibitor of DNA synthesis [2–5], but does so without toxicity at low concentrations [6]. This inhibition may occur by competitive inhibition of DNA polymerase [2, 3] or by terminal incorporation of ara-C into replicating DNA [7]; lethality may occur through a combination of both DNA synthetic inhibitory mechanisms [8, 9]. RNA and protein synthesis are less affected by ara-C [2, 10], but incorporation of ara-C into RNA may be the mechanism by which cellular lethality is produced [11].

In the process of examining the molecular [9] and cellular [12] effects of ara-C, differences were observed between the predicted and observed form of dose–survival curves *in vitro*. This difference was a consequence of incomplete removal of ara-C after a single rinsing procedure leading to continued exposure of treated cells to cytotoxic concentrations of the analogue.

MATERIALS AND METHODS

L-cells. L-cells, subline L60T$^-$ [13], were carried either in 250-ml spinner flasks (Bellco Laboratories) as a suspension culture or in 150-ml petri dishes as monolayer cultures. In either case, cells were cultured in α-MEM (Flow Laboratories) supplemented with 10% fetal calf serum (Gibco) (hereafter termed α-10). Cells were studied in the exponential phase of growth, that is, when cell numbers in suspension culture were from 5×10^4 to approximately 4×10^5 cells/ml.

Colony-forming assay. The number of cells with proliferative capacity sufficient to form a colony was assayed by plating 200–2000 cells in 5 ml of α-10 in 60-mm culture dishes (Falcon Plastics). Cells were allowed to attach to the culture dish for 1 hr prior to exposure to the drug. Appropriate drug concentrations were achieved by adding 20 μl from a more concentrated drug solution. Two separate rinsing procedures were used to end drug exposure. The simple rinse consisted of removing the drug-containing media from the culture dish, rinsing the dish with approximately 5 ml of sterile saline and finally replacing the media with fresh α-10. The extended rinse [14] consisted of first repeating the steps in the simple rinse; then, after an addition 1-hr incubation in fresh α-10, the dishes are again rinsed and the media replaced by fresh α-10. For this colony-forming assay and in all other experiments to be described, triplicate cell cultures were used at each drug concentration. Dishes were then left for 8 days in a CO_2 incubator during which time macroscopic colonies appeared; they were fixed and stained with a formalin, methylene blue mixture and counted.

Tritiated ara-C studies. Tritiated ara-C (sp. act. 20 Ci/m-mole New England Nuclear) was also used to quantitate the amount of drug remaining in cells after drug treatment and rinsing. Ara-C content of either the cells or the rinse(s) was determined by paper chromatography with a solvent of *sec*-butanol saturated water, as reported in the original quality control information obtained with the drug. The chromatogram was pressed to X-ray film (SB54 Kodak) and exposed for 1 week. After development of the film, corresponding areas on the chromatogram which were positive for ara-C were cut out, placed in counting fluid, and counted in a liquid scintillation counter.

RESULTS

Dose–survival studies on asynchronous cells. Cells were diluted to low density (200–2000 cells/dish) and exposed to concentrations of ara-C from 5×10^{-8} M to 5×10^{-4} M. Either 2 or 24 hr later, cells were rinsed by either the simple or extended technique. For either the 2- or 24-hr exposure (Fig. 1), with the simple wash, killing increased exponentially over the range of doses used. The shorter incubation served only to shift the exponential curve to the right so that equal cell killing required drug concentrations approximately a factor of 10 higher than necessary with the longer incubation.

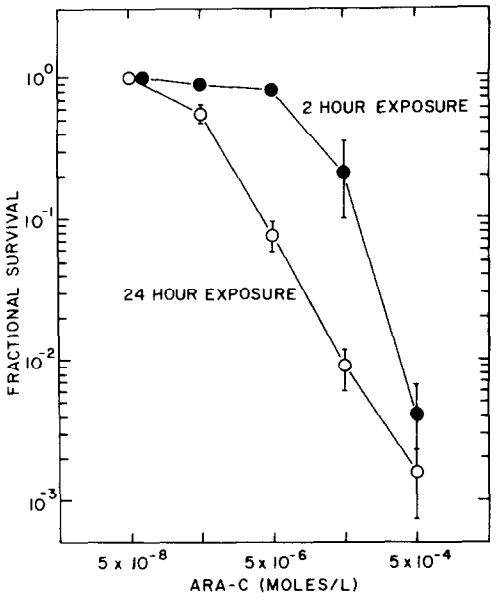

Fig. 1. Dose–survival curves for asynchronously growing L-cells treated for 2 or 24 hr with ara-C in which drug exposures were terminated by a simple wash. Means ± 1 S. D. are shown for the combined results of three to five separate experiments. Closed circles represent 2-hr exposures; open symbols, 24-hr exposures.

Thus, the plateau expected in a dose–survival curve for an S-phase specific agent [15] was not seen with a simple rinse for either the 2- or 24-hr exposures despite having chosen the 2-hr exposure specifically to be short enough so that only a single cohort of sensitive cells would be exposed, since 2 hr is much shorter than a generation time. The possibility that this result was due to drug persisting at cytotoxic levels despite the simple rinse prompted experiments using an extended washing procedure. For the 24-hr extended wash the slope of the dose–survival curve decreased, but the expected plateau was still not observed (Fig. 2). For the 2-hr extended wash only approximately 60 per cent of the cells were killed even at the highest dose used which is consistent with an S-phase limited cytotoxicity.

Drug persistence studies. To examine the possibility that drug was persisting intracellularly or was bound to the plastic culture dishes used and moving into the fresh media after the simple rinse to eventually reach cytotoxic levels, we performed experiments with tritiated ara-C and determined the biological activity of the rinses. One hundred cells were plated in 5 ml of α-10 in scintillation counting vials (Beckman), the radiolabelled drug was added at 10^{-5}, 10^{-6} or 10^{-7} M concentration and the cells were exposed for 2 hr. Both simple and extended rinses of these scintillation vials were performed and the radioactivity remaining in these rinses was determined. The results are shown in Fig. 3. In order to examine whether the radioactive counts were associated with ara-C after incubation, we chromatographed a sample of the rinse and over 90 per cent of the counts migrated to the position expected for ara-C. For the biologic assay for ara-C in the rinse, samples of the various rinses were added to 200 L-cells in petri dishes. The plating efficiency was then determined and compared to that obtained for known concentrations of ara-C using a 24-hr extended wash standard curve. These results are also shown in Fig. 3. For both assays, the amount of drug remaining in the second wash after all initial doses is approximately 0.5 to 1 per

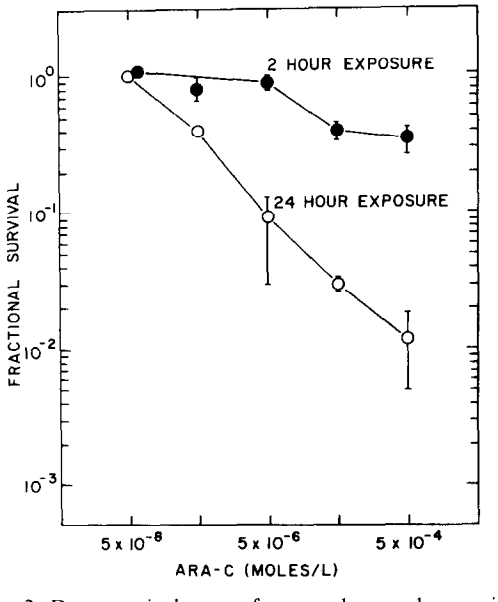

Fig. 2. Dose–survival curve for asynchronously growing L-cells treated for 2 or 24 hr with ara-C in which drug exposures were terminated by an extended wash. Means ± 1 S. D. are shown for the results of three to five separate experiments. Closed circles represent 2-hr exposures; open symbols, 24-hr exposures.

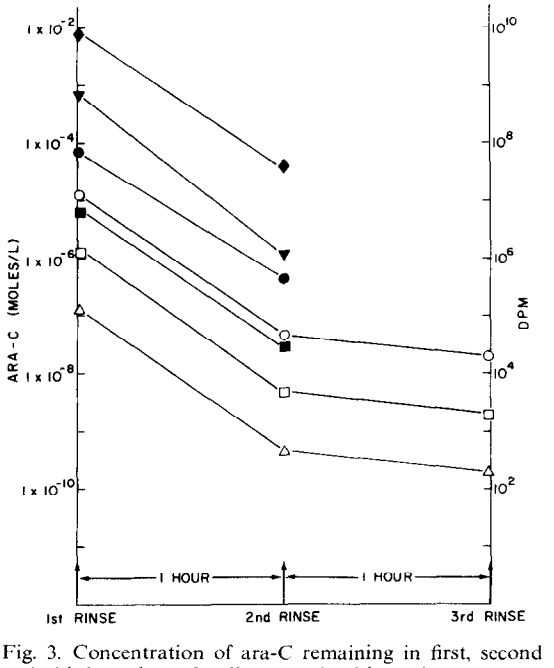

Fig. 3. Concentration of ara-C remaining in first, second and third washes of cells treated with various concentrations of ara-C. Open symbols represent remaining radioactive ara-C as determined by liquid scintillation counting; closed symbols represent remaining drug as determined by a cytotoxic assay.

Table 1. Plating efficiency of L-cells treated either with ara-C or with the media overlying ara-C-treated cells

Expt.	Initial drug concn (M)	Experimental conditions/results		
A		Extended rinse	3 × Saline	3 × PBS
	5×10^{-7}	0.60	0.31	0.36
	5×10^{-6}	0.44	0.23	0.15
	5×10^{-5}	0.20	0.08	0.13
B		Cell number	Rinse 2	
	5×10^{-7}	0	0.62	
		200	0.35	
	5×10^{-6}	0	0.50	
		200	0.46	
	5×10^{-5}	0	0.42	
		200	0.41	
C		Drug-treated cells after trypsinization	Rinse 2	
	5×10^{-7}	0.17	<0.005	
	5×10^{-6}	0.13	<0.005	
	5×10^{-5}	0.05	<0.005	

cent of the drug initially plated. Since a cytotoxic concentration is 5×10^{-7} M ara-C or higher, for an initial dose of 5×10^{-5} M or greater, cytotoxic concentrations persist after a simple rinse. In one experiment, a sample of the media was taken both 1 and 2 hr after the first wash in order to examine whether a 1-hr interval was sufficient to remove the drug remaining after the simple wash. The data in which 2 hr were allowed to elapse before the extended wash are identified in Fig. 3 as the "3rd rinse." As can be seen in Fig. 3, if more than 1 hr is allowed to elapse between the first and second wash, only slightly more drug can be removed by the second rinse.

In order to more precisely determine the location of the ara-C which persists, three additional experiments were performed using the biologic assay. In the first, a simple rinse was performed, but rather than rinsing singly with saline, three rinses were performed using phosphate buffered saline (PBS) or saline (Table 1A). As can be seen, immediate and thorough rinsing with either saline or PBS does not result in as high a plating efficiency as that obtained by the extended rinsing procedure.

In the second experiment, an assay of the biologic activity of extended rinses was performed either on plates containing 200 cells, or on control plates on which no cells were plated (Table 1B). Here it is seen that, although the presence of cells has some influence on the subsequent drug concentration in the overlying media, most of the drug is recovered from the culture dish. Finally, in the third experiment, cells were plated, exposed to drug-containing media, rinsed once and then trypsinized. One hr after trypsinization, the cells were rinsed again, and the biologic activity of the second rinse was measured (Table 1C). Here a result contrary to that seen in the previous experiment is found. For each drug concentration, the second rinse, obtained after the treated cells were removed from the plate in which they were treated, contains toxic concentrations of ara-C. The experiment, however, is complicated by the necessity for trypsinization, which apparently has toxicity for ara-C-treated cells, since the treated cells themselves show a lowered plating efficiency under these conditions.

DISCUSSION

A plateau in the dose–survival curve, expected for S-phase specific agents, was obtained when two washes were performed on low density monolayers treated with ara-C. If a simple rinse was performed, cell killing continued.

We have demonstrated that this is secondary to continued exposure to cytotoxic drug concentrations resulting from the release of from 0.5 to 1 per cent of the initial extracellular concentration of ara-C despite the initial wash. For concentrations greater than approximately 10^{-5} M, the amount of drug passing back into the media over a subsequent hour can exert significant lethality, and the effect of this residual drug must be considered for any experiment where the action of ara-C is terminated by a simple washing technique. This is especially important in studies where another agent is to be given sequentially with ara-C. It must be realized that, in such combinations where ara-C treatment precedes treatment with another agent, at least two rinses will probably have been performed, while when ara-C follows another agent, only one rinse after ara-C will have been done. One study in the literature [16] neglected this detail with the result that antagonism between ara-C and 2′-deoxy-5-fluorouridine (FUdR) was reported only when FUdR followed ara-C, thereby resulting in two rinses of the ara-C-treated cells. The conclusion may be valid, but similar differences in survival can be found in Figs. 1 and 2 where the only variable is the number of rinsing procedures.

Finally, as has been reported for actinomycin D [14] and demonstrated here for ara-C, the fact that cells are rinsed one or more times after drug exposure does not ensure that the treatment was administered as a "pulse". This probably occurs for other anticancer agents and must be considered when experiments *in vitro* of age response, progression delay, drug combination or other experiments assuming discrete exposure times are analyzed.

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