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Action of 1-β-D-Arabinofuranosyl-5-fluorocytosine on the Nucleic Acid Metabolism and Viability of HeLa Cells[1]

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Summary

Exposure of HeLa S-3 cells to 4×10^{-6} M 1-β-D-arabinofuranosyl-5-fluorocytosine (ara-FC)[2] arrested cell division. In the 1st 24-hr period, RNA and protein increased by a factor of about 2 while there was only a slight increase in the amount of DNA. The cell viability (defined as the capacity of a cell to grow out into a macroscopic clone) declined sharply following exposure to 1×10^{-6} M ara-FC for more than 1 generation time. These results strongly suggest a state of unbalanced growth similar to that previously observed with 5-fluorodeoxyuridine, excess thymidine and 1-β-D-arabinofuranosylcytosine (ara-C). The inhibition by ara-FC could be effectively prevented by simultaneous exposure of cells to a 100-fold excess of deoxycytidine, but not cytidine, thymidine, or deoxyuridine.

These results, strikingly similar to those previously obtained with ara-C, demonstrate that replacement of a hydrogen atom at the 5-position of the cytosine moiety of ara-C by the considerably more electronegative fluorine atom does not appreciably alter several important biochemical sites of inhibitory activity.

Introduction

1-β-D-Arabinofuranosylcytosine (ara-C) has been shown to be a growth inhibitor for several animal tumors (4, 5) and an effective inhibitor of DNA synthesis in mammalian cell culture (1, 9, 12). The inhibitory activity is related to the pyrimidine nucleoside structure containing the 1-β-D-arabinofuranosyl moiety. It was therefore considered important to study metabolic inhibition by structurally related compounds. 1-β-D-Arabinofuranosyl-5-fluorocytosine (ara-FC) has recently been synthesized (7). The studies reported in this paper provide some information on cell viability and nucleic acid synthesis in HeLa cells following treatment with ara-FC and make possible a comparison of the biologic activities of ara-FC and ara-C.

Materials and Methods

Experiments were carried out with HeLa cells of the S-3-5′ subline grown on Eagle's medium supplemented with 10% calf serum. The designation (5′) indicated that the cell population has been derived from a recloning operation. Details of the cell culture procedures were described elsewhere (9). Tests for contamination of the HeLa cell cultures with pleuropneumonia-like organisms (PPLO) were negative (6). The preparation and properties of ara-FC have been reported recently (7).

INCORPORATION OF LABELED PRECURSORS INTO NUCLEIC ACID AND PROTEINS. For studies involving the uptake of ^{3}H-labeled thymidine, uridine (^{3}H at carbon-5 position) and histidine into DNA, RNA, and protein, respectively, the following procedure was used: Equal numbers of cells (approximately 4×10^{5} cells/plate) were labeled for various times with thymidine-^{3}H (0.2 μc/ml, 1.9 c/mmole), uridine-^{3}H (1 μc/ml, 2.0 c/mmole), and histidine-^{3}H (1 μc/ml, 1.2 c/mmole). At the intervals stated, cells were washed 3 times with saline and were harvested by trypsinization. The trypsinized cells were filtered through a Millipore filter (23 mm in diameter; 0.45-μ pore size) according to the method of Kahan (8). The filter was washed with cold 5% trichloracetic acid. The radioactivity in the filter was determined with a liquid scintillation counter using standard procedures.

NUCLEIC ACID AND PROTEIN DETERMINATION. Nucleic acid and protein determinations were carried out by methods previously described (9, 10).

CELL AND COLONY COUNTS. Cell counts were performed with a Coulter Counter, model B. Plating for colony counts was carried out using 60-mm plastic Petri dishes. Control and ara-FC-treated plates prepared from trypsinized single cell suspensions were incubated for 14 days at 38°C. Colonies were fixed with methanol, stained with crystal violet, and counted after projection with a photographic enlarger.

Results

EFFECTS OF ara-FC ON CELL DIVISION. An experiment was carried out in which cells were exposed to various concentrations of ara-FC for 48 hr. The results are shown in Chart 1. The control cells showed a logarithmic growth with a doubling time of about 22 hr. Following exposure to 3.8×10^{-6} M ara-FC, the cell number increased slightly for 5 hr and then remained stationary up to 48 hr. When the cells were exposed to 3.8×10^{-7} M ara-FC, the cell number remained stationary up to 30 hr and recovered partially up to 60% of the control value at 48 hr.

EFFECT OF SEVERAL PYRIMIDINE NUCLEOSIDES ON INHIBITION BY ara-FC. Cells were plated for 24 hr before the compounds listed in Table 1 were added to the culture medium. The results in the table show that 3.8×10^{-6} M ara-FC stopped the growth of the cell population. This inhibition was effectively prevented

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[2] The following abbreviations are used: ara-C, 1-β-D-arabinofuranosylcytosine; ara-FC, 1-β-D-arabinofuranosyl-5-fluorocytosine.

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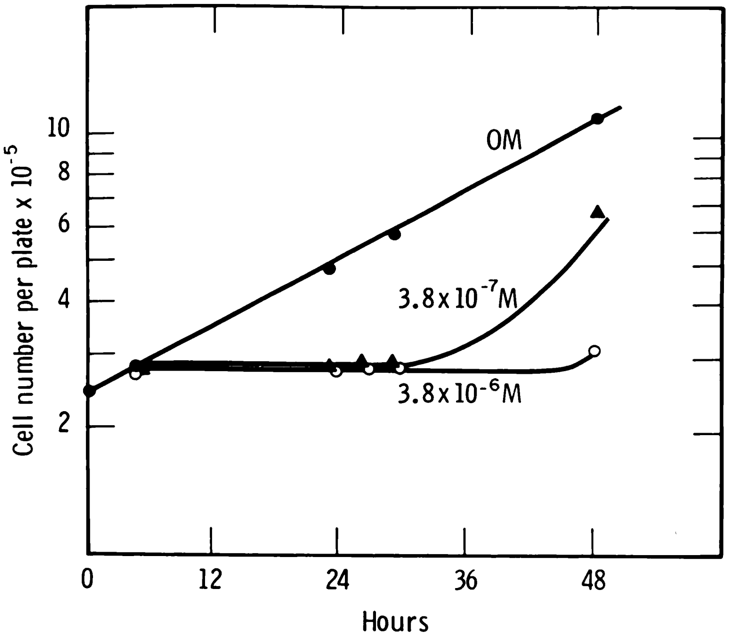

CHART 1. Changes in the number of cells/plate following exposure of HeLa S-3 cells to several concentrations of ara-FC. (The curve labeled *OM* refers to untreated control cells.)

TABLE 1

EFFECT OF SEVERAL PYRIMIDINE NUCLEOSIDES ON THE ACTION OF 1-β-D-ARABINOFURANOSYL-5-FLUOROCYTOSINE (ara-FC)

Ara-FC	Nucleoside	No. of cells × 10^{-4}		
		0 hr	24 hr	48 hr
−		8.4	16.2	34.5
+[a]			7.6	5.9
+	Deoxycytidine[b]		15.2	32.6
−	Deoxycytidine		16.6	38.0
+	Deoxyuridine		8.3	6.4
−	Deoxyuridine		16.1	34.6
+	Thymidine		8.0	5.7
−	Thymidine		11.5	26.8
+	Cytidine		7.8	7.0
−	Cytidine		15.6	37.3

[a] + refers to 3.8 × 10^{-6} M ara-FC.

[b] Deoxycytidine, deoxyuridine, thymidine, and cytidine added at 1.8 × 10^{-4} M. Cells were plated 24 hr before the indicated compounds were added to the medium. At the indicated time, the cell number/plate was determined.

by the addition of 3.6 × 10^{-4} M deoxycytidine. The addition of cytidine, thymidine, and deoxyuridine to the ara-FC-containing medium did not prevent the growth inhibition.

EFFECT OF ara-FC ON NUCLEIC ACID AND PROTEIN SYNTHESIS. Measurements of the total DNA, RNA, and protein content of HeLa S-3 cells were carried out at different times after addition of 3.8 × 10^{-6} M ara-FC. Chart 2 shows the unbalanced growth induced by exposure to ara-FC. The total DNA in the culture increased by only about 30% in the presence of ara-FC, whereas the RNA and protein/plate increased almost by a factor of 2.

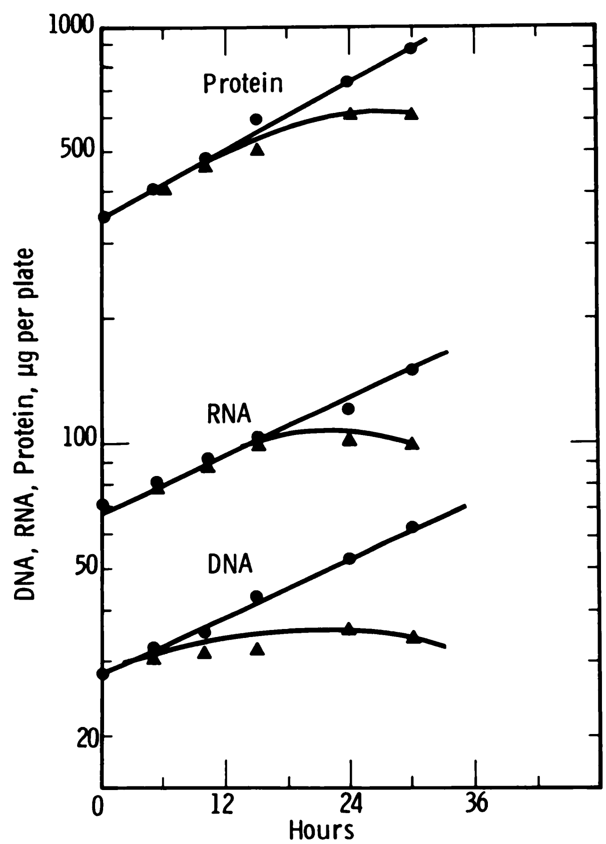

CHART 2. Changes in the amount of DNA, RNA, and protein/plate as a function of time following exposure of HeLa S-3 cells to 3.8 × 10^{-6} M ara-FC. ●, control; ▲, ara-FC.

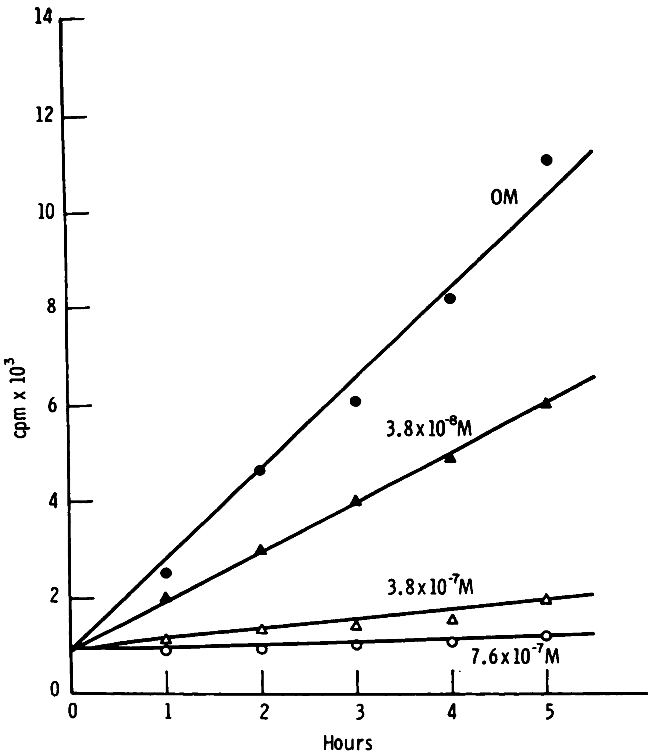

CHART 3. Incorporation of thymidine-^{3}H into DNA of HeLa S-3 cells exposed to various concentrations of ara-FC.

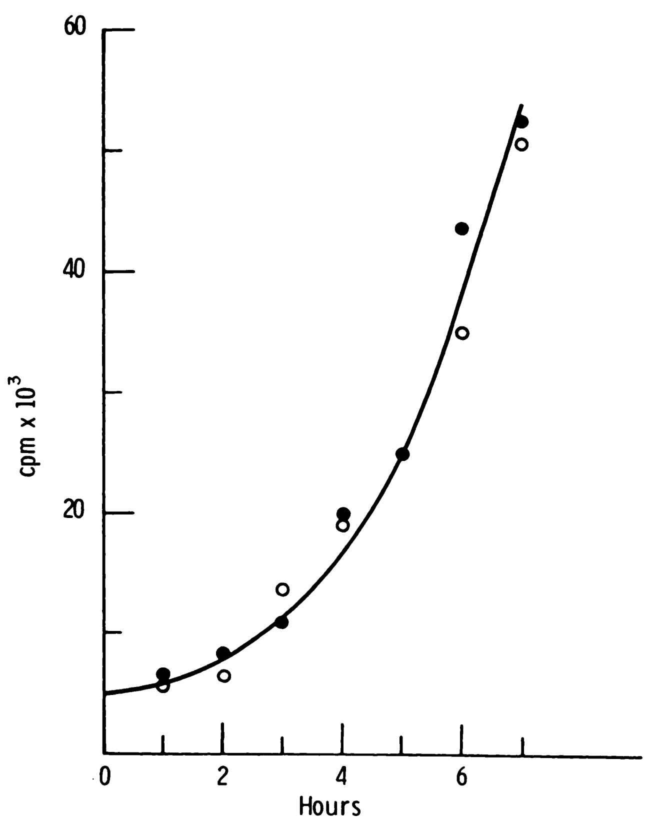

CHART 4. Incorporation of uridine-^{3}H into RNA of HeLa S-3 cells exposed to ara-FC. ●, control; ○, 3.8 × 10^{-6} M ara-FC.

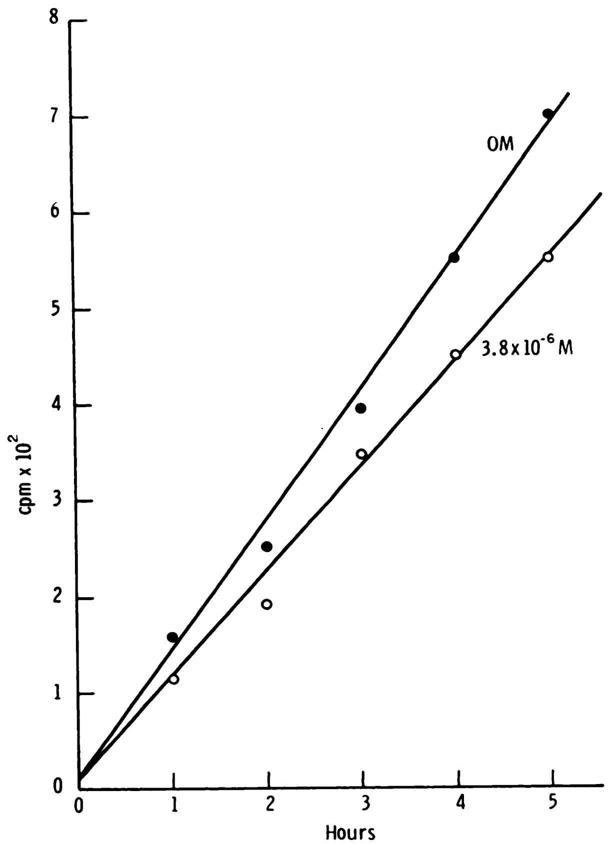

CHART 5. Incorporation of histidine-^{3}H into protein of HeLa S-3 cells exposed to ara-FC.

INCORPORATION OF SOME LABELED PRECURSORS INTO DNA, RNA, AND PROTEIN IN THE PRESENCE OF ara-FC. As shown above, exposure to ara-FC resulted in an unbalanced growth of HeLa cells. It was of interest to determine whether the utilization of selected ^{3}H-containing precursors would lead to a similar conclusion. Chart 3 shows the effect of various concentrations of ara-FC on the incorporation of thymidine-^{3}H into cellular DNA. At 7.6 × 10^{-7} M, thymidine-^{3}H incorporation into DNA was almost immediately and completely stopped, whereas uridine-^{3}H and histidine-^{3}H incorporation into RNA and protein respectively were very slightly affected (Charts 4, 5)

EFFECT OF ARA-FC ON CELL VIABILITY. The cell viability (defined in terms of the capacity of a cell to grow out into a macroscopic colony) is shown in Chart 6 as a function of time of exposure to ara-FC. Cell plating efficiencies (defined here as the ratio of total colonies formed to the number of single cells initially inoculated/plate) were generally 0.7–0.75 for HeLa cells. The general pattern of the survival curves was similar to those obtained by treatment with ara-C, excess thymidine or 5-fluorodeoxyuridine (3, 9–11). Exposure for a period greater than 1 generation time yields a markedly lowered viability.

Discussion

The present study shows that ara-FC is an effective inhibitor of DNA synthesis in HeLa S-3 cells (Charts 2, 3). The results in Chart 2 also demonstrate that the continued synthesis of RNA

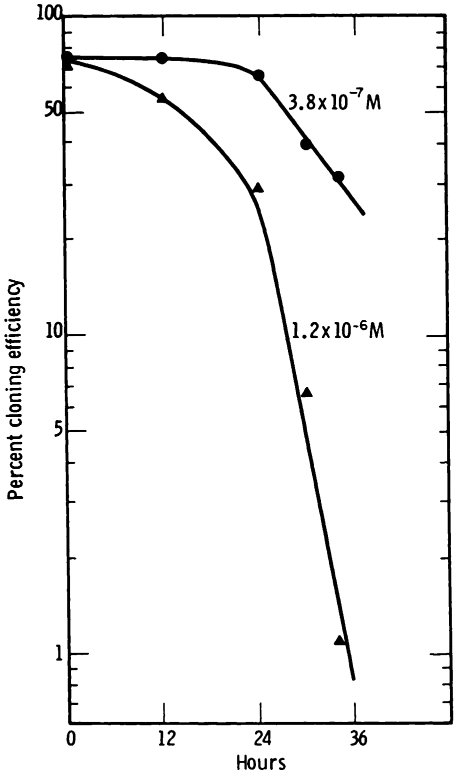

CHART 6. The loss of reproductive capacity following exposure of HeLa S-3 cells to 2 concentrations of ara-FC. Single cells suspensions were incubated for 14 days and macroscopic colonies were counted. Each *point* represents the average of 6 replicate plates.

and protein in the presence of ara-FC leads to a state of unbalanced growth analogous to that induced by ara-C (9), 5-fluorodeoxyuridine (3, 11), and excess thymidine (10). The viability curve exhibits a shoulder at low exposure times and sharply reduced cell viability for an exposure period longer than 24 hr (Chart 6).

The reversal studies with several pyrimidine nucleosides showed that only deoxycytidine was able to prevent inhibition of cell growth by ara-FC (Table 1). The results in Table 1 are similar to those obtained with ara-C in our previous studies (9). Previous studies showed that ara-C inhibited the reduction of the cytidylate to the deoxycytidylate moiety in murine lymphoblasts and neoplastic mast cells (1).

The results described above demonstrate that effects of ara-FC and ara-C on several important metabolic activities of HeLa S-3 cells are strikingly similar. These include: (*a*) continued synthesis of RNA and protein accompanied by a sharp decrease in the rate of DNA synthesis (unbalanced growth), and (*b*) a sharp decrease in the capacity of single cells to grow out into macroscopic clones following exposure to these compounds for more than 1 division time. Replacement of a hydrogen atom at the 5-position of the cytosine moiety by the considerably more electronegative fluorine atom does not significantly affect metabolic activities tested to date of the HeLa S-3 cells.

The incorporation of labeled ara-C to a small extent into the DNA and RNA of L 5178 Y cells and mouse fibroblasts, Strain L-929 has recently been reported (2, 12). Silagi has postulated that the incorporation of very small amounts of ara-C into DNA may underlie the observed inhibition of DNA synthesis and result in loss of cell viability (12). This hypothesis may not apply to the experimental conditions described above. Cell viability was essentially unimpaired (Chart 6) following exposure to 3.8×10^{-7} M ara-FC for less than 24 hr while DNA synthesis sharply decreased during this period (Chart 1). The relative importance of this postulated mechanism and that proposed by Chu and Fischer (1) for the inhibitory effects observed in cell culture requires extensive additional study with both ara-C and ara-FC.

Acknowledgments

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References

1. Chu, M. Y., and Fischer, G. A. A Proposed Mechanism of Action of 1-β-D-arabinofuranosylcytosine as an Inhibitor of the Growth of Leukemic Cells. Biochem. Pharmacol., *11:* 423–30, 1962.
2. ———. Comparative Studies of Leukemic Cells Sensitive and Resistant to Cytosine Arabinoside. *Ibid.*, *14:* 331–41, 1965.
3. Eidinoff, M. L., and Rich, M. A. Growth Inhibition of a Human Tumor Cell Strain by 5-fluoro-2′-Deoxyuridine: Time Parameters for Subsequent Reversal by Thymidine. Cancer Res., *19:* 521–24, 1959.
4. Evans, J. S., Musser, E. A., Bostwick, L., and Mengel, G. D. The Effect of 1-β-D-arabinofuranosylcytosine Hydrochloride on Murine Neoplasms. *Ibid.*, *24:* 1285–93, 1964.
5. Evans, J. S., Musser, E. A., Mengel, G. D., Forsblad, K. R., and Hunter, J. H. Antitumor Activity of 1-β-D-arabinofuranosylcytosine Hydrochloride. Proc. Soc. Exptl. Biol. Med., *106:* 350–53, 1961.
6. Fogh, J., and Fogh, H. A Method for Direct Demonstration of Pleuropneumonia-Like Organisms in Cultured Cells. *Ibid.*, *117:* 899–901, 1964.
7. Fox, J. J., Miller, N., and Wempen, I. Nucleosides XXIX. 1-β-D-Arabinofuranosyl-5-fluorocytosine (FCA) and Related Arabino Nucleosides. J. Med. Chem., *9:* 101–5, 1966.
8. Kahan, F. M. Purification and Measurement of Microgram Amounts of Radioactive Nucleic Acids and Proteins from Animal Cells in Tissue Culture. Anal. Biochem., *1:* 107–26, 1960.
9. Kim, J. H., and Eidinoff, M. L. Action of 1-β-D-Arabinofuranosylcytosine on the Nucleic Acid Metabolism and Viability of HeLa Cells. Cancer Res., *25:* 698–702, 1965.
10. Kim, J. H., Kim, S. H., and Eidinoff, M. L. Cell Viability and Nucleic Acid Metabolism after Exposure of HeLa Cells to Excess Thymidine and Deoxyadenosine. Biochem. Pharmacol., *14:* 1821–29, 1965.
11. Reukert, R., and Mueller, G. C. Studies on Unbalanced Growth in Tissue Culture. I. Induction and Consequences of Thymidine Deficiency. Cancer Res., *20:* 1584–91, 1960.
12. Silagi, S. Metabolism of 1-β-D-Arabinofuranosylcytosine in L Cells. *Ibid.*, *25:* 1446–53, 1965.