Some Effects of d-Arabinosyl Nucleosides on Polymer Syntheses in Mouse Fibroblasts

ALICE DOERING, JOAN KELLER, AND SEYMOUR S. COHEN

Department of Therapeutic Research, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Summary

The effects of 1-β-D-arabinofuranosylcytosine and of 9-β-D-arabinofuranosyladenine have been studied on the growth and multiplication of mouse fibroblasts in suspended cell culture. Both compounds permit a considerable enlargement of the cells but sharply inhibit multiplication. The effects of these agents on the syntheses of DNA, RNA, and protein in these cells have also been examined. Both chemicals markedly inhibit DNA synthesis and reduce the cloning capacity of the cultures. No significant effect of either compound has been detected on RNA synthesis. A slight inhibition of protein synthesis has been observed with arabinosyladenine. After prolonged inhibition of cell multiplication by either compound, washed cells, incubated in the normal medium supplemented with thymidine-2-14C, were able to synthesize DNA at an approximately normal rate for 6 hr. After this time, DNA synthesis stopped abruptly and cell division occurred in 50–80% of the cells. It has been concluded that inhibition of DNA synthesis and of cell multiplication by D-arabinosyl nucleosides is probably not attributable to irreversible lesions in the DNA resulting from terminal addition of D-arabinosyl nucleotides.

Introduction

Several synthetic nucleosides containing D-arabinose as the sugar component instead of D-ribose or D-deoxyribose have been shown to be toxic, indeed lethal, to various types of cells. The chemical, biochemical, and biologic literature concerning these compounds has recently been reviewed (8). One of these, ara-C, is highly inhibitory to the multiplication of mammalian cells and to the multiplication of animal viruses containing DNA. These inhibitory effects demonstrable in tissue cultures and in intact animals are prevented specifically by the exogenous addition of deoxycytidine. This specific prevention of inhibition by the normal nucleoside and the relative inactivity of D-arabinosyluracil was considered to suggest that ara-C produces a specific lesion in DNA synthesis, presumably the specific inhibition of ribonucleotide reductase (6). With isolated enzyme systems derived from Escherichia coli, synthetic ara-C nucleotides were not detectably incorporated into newly formed RNA or DNA, nor were these compounds markedly inhibitory in these synthetic reactions (5). More recently ara-CDP or ara-CTP were not observed to be effective inhibitors of the purified ribonucleotide reductase of rat tumor cells in the conversion of CDP to dCDP (Moore and Cohen, unpublished data).

Two independent reports have appeared suggesting that small amounts of ara-C are incorporated into DNA in cultured animal cells (7, 17). Although these papers do not present convincing proof of such incorporation, it was nevertheless conceivable that such incorporation might occur and indeed be lethal, if, for example, the addition of a single arabinosyl nucleotide prevented subsequent DNA synthesis in separate chromosomes. The production of chromosome breaks in cells treated with ara-C and ara-A has been described (15).

Similar problems concerning a mechanism of inhibition arise in connection with the toxicity of ara-A. This compound was observed to be severely inhibitory of DNA synthesis as well as lethal to certain purine-requiring strains of E. coli (11, 12). It is also severely toxic to animal cells in culture (2, 8) as well as inhibitory to the development of tumors in mice (3). We have shown that, to the level of sensitivity with which we have experimented with ara-C nucleotides, nucleotides of ara-A are not incorporated into RNA or DNA in purified bacterial cell-free systems (8). On the other hand, the di- and triphosphates of ara-A are markedly inhibitory of a mammalian ribonucleotide reductase in the conversion of CDP, UDP, GDP, and ADP to the deoxynucleoside diphosphates (Moore and Cohen, unpublished data). Furthermore, it has recently been reported that ara-ATP is an effective inhibitor of the DNA polymerase of mouse tumor cells, a result to which is ascribed the effectiveness of ara-A in inhibiting tumor development in mice (18). Although no incorporation of ara-A into DNA of bacteria or animal cells has been detected (8), it was conceivable that terminal addition could again be responsible for the observed lethality of this nucleoside.

In connection with our studies on the nature of lethality in these and other systems, i.e., thymineless death, streptomycin, etc., we have undertaken to determine the gross patterns of polymer synthesis in suspended cultures of mouse fibroblasts (L
cells) in the presence of the inhibitory d-arabinosyl nucleosides. After showing that ara-C and ara-A appeared to effect DNA synthesis relatively specifically, we studied the abilities of the inhibited cells, after washing to remove inhibitor, to synthesize DNA, as measured by thymidine incorporation. It was found that such cells are capable of an almost normal initial rate of thymidine incorporation, i.e., neither the DNA templates nor the DNA polymerase are irreversibly prevented from reinitiating DNA synthesis as a result of extended inhibition by the ara nucleosides.

Materials and Methods

Mouse fibroblasts (L cells) were derived from the stocks of Dr. John Littlefield of the Massachusetts General Hospital. They were grown in suspension as previously described (13). The medium was slightly modified from that of Eagle and in addition to containing streptomycin and penicillin was supplemented with 10% fetal calf serum. Cell counts were obtained with the Coulter electronic counter Model B. The distribution of cell sizes were also obtained with this instrument. All experiments were begun with cells in the log phase of growth. Early cultures of our cells were found by Dr. H. Morton to be negative for PPLO infection; later cultures were also negative in this respect according to Dr. L. Hayflick.

To estimate the viability of the cultures, aliquots were diluted to 1000 cells/ml, 0.1 ml was added to 3 ml of a bicarbonate-buffered medium in a 60-mm Petri dish that had been warmed to 37°C and equilibrated in a desiccator with an air-5% CO2 mixture. After an even distribution of the cells, the dish was returned to the gassed desiccator, and incubated for a week to form colonies. Cells harvested in log phase had a plating efficiency of 70–80%.

ara-C was a gift from the Upjohn Company; ara-A was synthesized by the method of Glaudemans and Fletcher (10) or was obtained from the Cancer Chemotherapy National Service Center, Bethesda, Maryland; ara-Hx was prepared by the enzymatic deamination of ara-A (8) and recrystallized twice from water; thymidine-2-14C at 30 μC/μmole was purchased from the New England Nuclear Corporation and diluted with thymidine-4C to the desired specific activity.

For estimations of nucleic acid content, aliquots containing approximately 4.5 × 10^6 cells were sedimented 10 min at 1500 rpm, washed with 5 ml of PBS, and frozen until the desired samples were collected. Pellets were washed again with 2 ml 3% cold perchloric acid. After 10 min at 0°C, the precipitate was sedimented (2000 rpm for 10 min), and the supernatant fluid was removed. After hydrolysis overnight at 37°C in NaOH, the chilled samples were acidified, and the precipitate was separated by centrifugation. RNA in the supernatant fluid was estimated by the Bial reaction with orcinol. DNA was extracted from the precipitate by heating with 3% perchloric acid and estimated by the diphenylamine reaction (4).

In estimating protein content, aliquots of 2–5 × 10^6 cells were centrifuged, washed twice with PBS, and frozen. The pellets were dissolved in 1 ml of 0.1 N NaOH and analyzed by the method of Lowry et al. (14).

In the measurement of the radioactivity of the cells after incorporation of thymidine-2-14C, aliquots containing 4 × 10^5 cells were added to chilled PBS and centrifuged at 1500 rpm for 10 min. The cells, resuspended in 1 ml of cold PBS, were precipitated with 3 volumes of cold 3% perchloric acid. After 10 min at 0°C, the cells were filtered on a Millipore filter and washed twice with 10 ml H2O. The filters were air-dried, immersed in 6 ml of PPO-PPOPOP-toluene mixture, and counted in a Packard Tri-Carb liquid scintillation counter. Under these conditions, the efficiency of counting was estimated to be 60% as compared to the counting of free thymidine added to the scintillation fluid in this system.

ADDITION OF COMPOUNDS. Since the medium containing calf serum had an appreciable adenosine deaminase activity, the compounds were added immediately prior to the beginning of the experiment. Heating of the serum at 56°C for 30 min did not affect the deaminase. Ara-C (2 × 10^-4 M) was filtered and added directly (1:50 or 1:100 dilution) in the required amount to the culture. Ara-A was inhibitory in the system at 2 × 10^-4 M but could only be dissolved at 2 × 10^-3 M. The compound was therefore preweighed, sterilized with ethanol, and dried in vacuo. The dried powder was then incorporated directly into the medium and dissolved at 37°C. An alternative method resorted to solution to the desired concentration in serum-free medium, followed by filtration through a Millipore filter.

Results

INHIBITION BY ara-C. The kinetics of growth in our spinner cultures are highly reproducible. Paired growing cultures diluted in fresh medium to about 2 × 10^4 cells/ml grew in exponential phase at closely similar rates to 10^5 cells/ml, and then began to slow their rate of growth. Compounds were added when the cultures were in the range of 4 to 8 × 10^4 cells/ml. In experiments involving incorporation of thymidine-2-14C, this compound was used at 4 × 10^-4 M containing 0.12 μC/ml of culture.

In Chart 1 is seen the effect of 2 × 10^-5 M ara-C on the culture whose chemical and other parameters are presented in Chart 2. It can be seen that the size distribution of the cells in the normal culture does not change detectably whereas that containing ara-C begins to shift at about 6 hr and is markedly distorted at 20 hr. The increase in cell number in the culture (Chart 2) has stopped abruptly at 6 hr, at which time cloning efficiency falls precipitously. In the terms of the microbiologist, measuring cell viability by the ability to form colonies, a considerable proportion of the cells are dead. Examined in the electron microscope, with the kind cooperation of Dr. M. Mass of this Department, the cells are indeed enlarged and the large central nuclei appear to have masses of chromatin somewhat condensed on or near the nuclear membrane. Nevertheless, the nuclei do not appear clearly damaged nor is there detectable vacuolization or other easily observable lesions in the cytoplasm.

When, after about 24 hr such inhibited cells are washed and resuspended in fresh medium, a significant percentage of the cells do not enter into cell division but remain quite large, permanently distorting the pattern of size distribution. It does not seem possible to determine the percentage of "dead" cells from the percentage within this abnormal size class both because many cells lyse after transferring the culture in normal media and the residual fraction of irreversibly large cells also merges in size with the normal pattern.

RNA and protein synthesis were not inhibited at all in this...
CHART 1. Size distribution curves of L cell suspensions growing in the presence or absence of d-arabinosylcytosine at $2 \times 10^{-4}$ M. Curves taken from graphs traced by the Coulter size distribution plotter have been normalized to represent the same numbers of cells at different times in the 2 cultures. ●, −2 hr; ○, 0.5 hr; ×, 6 hr; △, 22 hr.

CHART 2. Multiplication and syntheses of RNA, DNA, and protein in L cell suspensions in the presence or absence of d-arabinosylcytosine at $2 \times 10^{-4}$ M. The units are presented next to the curves. ×—×, Curves for paired cultures to which the d-arabinosyl nucleosides have been added. ○—○, Comparative controls.
Chart 3. Size distribution curves of L cells growing in the presence or absence of D-arabinosyladenine at 2 × 10⁻⁴ M. ●, −3.5 hr; ○, 0.5 hr; ×, 4.5 hr; Δ, 20.5 hr.

Chart 4. Multiplication and syntheses of RNA, DNA, and protein in L cells in the presence or absence of D-arabinosyladenine at 2 × 10⁻⁴ M. The units are presented next to the curves. X—X, Curves for paired cultures to which the D-arabinosyl nucleosides have been added. ○—○, Comparative controls.

System, as presented in Chart 2. However, the estimations of DNA reveal a significant inhibition of synthesis of this component by ara-C shortly after addition. Studies of thymidine incorporation into an acid-insoluble fraction, presumably DNA, have indicated an almost total inhibition for up to 6 hr. After this time, the incorporation of thymidine at 2 × 10⁻⁴ M and 4 × 10⁻⁴ ara-C can be up to 40 and 20%, respectively, of the control rate.
INHIBITION BY ara-A. Ara-A is a much less active inhibitor in this system and must be added at $2 \times 10^{-4}$ M to be effective. As presented in Chart 3, the compound produces changes in cell size very similar to those observed with ara-C. Examination in the electron microscope has revealed cytologic effects superficially indistinguishable from those noted with ara-C.

In Chart 4, it can be seen that there is some inhibition of protein synthesis although only a minimal, if at all significant, change can be found in RNA production. On the other hand, the inhibition of increase of cell number and of DNA is far more severe and the loss of cloning ability also approaches effects observed with ara-C. In studies on thymidine incorporation, $2 \times 10^{-4}$ M ara-A can produce a sharp inhibition in DNA synthesis for several hr but, as with ara-C, it can resume at a reduced rate, up to 50% of the control.

In these cultures, ara-A is rapidly deaminated and ara-Hx is found almost quantitatively in the medium after 24 hr. Ara-Hx itself at $2 \times 10^{-3}$ M produces an inhibition of L cells approximately comparable to that of ara-A at $2 \times 10^{-4}$ M. Thus, the effects observed seem substantially that of ara-A, although the ara-Hx generated perhaps contributes significantly to some extent. In recent experiments, an inhibitor of mammalian adenosine deaminase, 9-(3'-hydroxypropyl)-6-aminopurine (16), was kindly provided by Dr. H. Schaeffer of the University of Buffalo. This compound, although not detectably toxic itself, sharply potentiates the lethality of ara-A and also seems to decrease cell enlargement somewhat. Thus, it may well be that the in vivo deamination of ara-A to ara-Hx reduces the apparent effect on protein synthesis.

RESUMPTION OF DNA SYNTHESIS AFTER INHIBITION BY ara-C AND ara-A. Cultures were inhibited for 24 hr by $4 \times 10^{-8}$ M ara-C and by $2 \times 10^{-4}$ M ara-A. At this time the cells were centrifuged lightly, and the medium was replaced by fresh medium which lacked the inhibitory ara nucleoside and contained $4 \times 10^{-8}$ M thymidine-2-14C. Aliquots were removed at frequent intervals over another 24-hr period. In a typical experiment, data were obtained on the change in cell number and the distribution of cell size in the cultures; these are presented in Chart 5. A normal control, containing initially fewer cells, was used to determine the effects of manipulation, medium replacement, and of added...
Discussion

The demonstration that an animal cell is killed by a given agent is more difficult to establish than that readily effected with bacteria. The ability to form colonies is tested readily in an 18-hr incubation in the case of bacteria, but requires a week with L cells. In the latter instance, or with other animal cells, cloning is a relatively new art and cloning efficiencies are rarely as high as we have somehow managed to attain with the L cells. Furthermore, most bacteria are programmed for division as an ordinary concomitant of cell growth (“balanced growth”), whereas many animal cells have either lost the capacity for division as an irreversible phenomenon or as a natural concomitant of differentiation for specialized function. In the case of many animal cells, an inability to clone would scarcely be accepted as a criterion of cell death, e.g., nerve cells, even though this is certainly the most common criterion of death in microbiology. Despite the relative dearth of cloning data of this type in the study of the toxicity of compounds on appropriate animal cells, such a criterion might well be used more commonly in studies of cancer chemotherapy as it is in microbiology. We are impressed not only by the feasibility of such cloning tests of toxic agents with appropriate cells but also by the ease of use and information proved by the Coulter counter in a concomitant dissection of inhibition and cell growth. On the other hand, the study of toxic agents on animal cells in culture undoubtedly is far more difficult than is a comparable study in bacteria. Nevertheless, ara-C has not been found to be toxic in bacteria, and the opportunity for pinpointing the sites of development of cell pathology are clearly greater for animal cells than for bacteria.

The data presented in this paper affirm the now well-known effects of ara-C in inhibiting DNA synthesis and cellular multiplication. At the concentrations we have used, i.e., $2 - 4 \times 10^{-4} \text{M}$, the inhibition of cellular multiplication appears more severe than that of DNA synthesis. In our studies, in which cellular arrest has been essentially complete, lethality has been far from complete, approaching 50% after 24 hr in some experiments and in that described in Chart 5, markedly less than that.

Similar results were observed with ara-A at a 50-fold higher concentration, although this compound seemed to have a slight inhibitory effect on protein synthesis as well as on DNA synthesis. A clearer pattern of effects of ara-A itself can be expected to emerge when the use of this compound is combined with that of an appropriate inhibitor of adenosine deaminase. Nevertheless, the agent, possibly effective in conjunction with derived ara-Hx, does present a pattern of somewhat greater lethality in our experiments, as well as severe inhibition of cellular multiplication.

In this light, the data of Chu and Fisher (6) and of Silagi (17) suggesting incorporation of ara-C into DNA may be considered.

Chart 6. Incorporation of thymidine-2-14C in L cells previously incubated for 18 hr in the presence or absence of d-arabinosyl nucleosides (see Chart 5). Thymidine-2-14C is $4 \times 10^{-4} \text{M} (0.12 \mu \text{c/ml}).$
It is our opinion that these experiments, for reasons described elsewhere (8), can not yet be considered as definitive evidence of incorporation into DNA. It appears to us that the isolation of well-characterized 3'-ara-CMP from the nucleic acid of a cell exposed to ara-C would be a convincing demonstration of such incorporation into a polynucleotide chain, but this has not yet been attempted.

Nevertheless, an incorporation of an ara nucleoside into DNA is possible in principle and could account for the inhibition of DNA synthesis and lethality if the incorporation occurred at the ends of DNA molecule and prevented subsequent DNA synthesis. Such a result is in fact implied by the data of Chu and Fisher (7), who reported a very low degree of incorporation in the DNA. The experiment described in this paper shows that a prior inhibition of DNA synthesis with ara nucleosides does not prevent a subsequent rapid rate of DNA synthesis in washed cells, even greater than in the control, for 6 hr. This result suggests strongly that the ends of the DNA molecules of the inhibited cells did not contain ara-C or ara-A which might prevent subsequent addition of deoxyribonucleotides. Furthermore, this DNA synthesis proceeded at these high rates in cultures containing significant numbers of "dead" cells, a phenomenon also observed readily in bacterial cultures which had suffered thymineless death (1) or killing by ara-A to 1% survival or less (9). Thus, the cause of lethality must be sought elsewhere than in the incorporation of ara compounds to the postulated damaged ends of DNA.

References


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Alice Doering, Joan Keller and Seymour S. Cohen


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