Antiproliferative Effects of 9-β-D-Arabinofuranosyladenine in a Mammalian Cell Line Devoid of Adenosine Deaminase Activity

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The antiviral drug arabinosyladenine inhibited cellular growth and DNA synthesis in an adenosine deaminase-negative cell line (B-mix K-44/6). Use of adenosine deaminase-containing calf serum in the culture medium reversed the drug-induced inhibition and resulted in a recovery of both mitosis and the rate of DNA synthesis.

The fraudulent nucleoside 9-β-D-arabinofuranosyladenine (ara-A*) possesses antiproliferative activity against cells in culture and has some activity against transplantable animal tumors.[1-3] The drug also is active against a broad spectrum of DNA viruses including herpesviruses. It is active in cell cultures,[4,5] in experimental animals[4,6] and clinical studies have indicated its usefulness against herpetic keratitis[7] and disseminated herpes zoster.[8] The topical use of ara-A for the treatment of herpes labialis currently is being explored at The University of Michigan, School of Dentistry.

The present interest in treating herpesvirus infections with ara-A has arisen largely because the drug does not produce serious toxicity in animals[9] or man [8,10,11] when used in its antiviral dose range. We have been studying the biochemical basis for the selective toxicity toward herpesviruses and have found that ara-A inhibits viral DNA synthesis to a greater extent than host cell DNA synthesis.[12,13] On the basis of this in vitro work, we have postulated that the preferential sensitivity of viral DNA synthesis to ara-A is related to the high therapeutic index noted in vivo.[14]

Another factor also may be involved in the low toxicity of the drug. Ara-A is degraded by adenosine deaminase to ara-H[1,2] which is considerably less cytotoxic than ara-A.[12-16] Consequently, the deamination of ara-A in tissues susceptible to the action of the drug may be an additional mechanism responsible for the relatively low toxicity of ara-A observed in vivo.[17,18] This assumes, however, that once ara-A is metabolized to ara-H, cells can recover from the inhibitory effects. Inhibitors of adenosine deaminase recently have become available.[19-21] When combined with such an inhibitor, ara-A is considerably more cytotoxic than when given alone.[13,18,22,23] The implication is that in the absence of an inhibitor, cells do recover from the effects of ara-A as it is being deaminated to ara-H. We have tested this assumption directly by utilizing a Rous sarcoma virus-transformed line of embryonic rat cells (B-mix K-44/6)[24] which is devoid of adenosine deaminase activity.[25,26] We have observed that the cell line will grow in medium supplemented either with calf serum—which contains adenosine deaminase[16,27-29]—or with horse serum—which does not.[27,29] Consequently, we have been able to compare the effects of ara-A in cell culture systems that do and do not deaminate ara-A without recourse to the use of an adenosine deaminase inhibitor.

This investigation was supported by Grant DE-02731 from the National Institute of Dental Research. J.C.D. was supported in part by Grant GM-02010 from the National Institute of General Medical Sciences.

A portion of this material was presented before the American Society for Pharmacology and Experimental Therapeutics, Atlantic City, New Jersey, April 1973.

Received for publication October 11, 1976.

Accepted for publication December 6, 1976.

* Abbreviations used are: Ade, adenine; ara-A, 9-β-D-arabinofuranosyladenine; ara-C, 1-β-D-arabinofuranosylcytosine; ara-H, 9-β-D-arabinofuranosylhypoxanthine; EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline (see ref. 30); HSV, herpes simplex virus; Hyp, hypoxanthine; PEI, polyethyleneimine; PPO, diphenyloxazole; and TCA, trichloroacetic acid.

Materials and Methods

DRUGS AND CHEMICALS.—Ara-A was provided through the courtesy of Dr. H. Machamer of Parke, Davis and Co., Detroit, Mi. [8-^{14}C]-ATP (50 mCi/mmole), [methyl-^{3}H]thymidine (6.7 Ci/mmole, and [5-^{3}H]uridine (20 Ci/mmole) were purchased from New England Nuclear, Boston, Ma.

CELL CULTURE SYSTEMS.—B-mix K-44/6 Rous sarcoma virus-transformed rat cells were the gift of Dr. Jan Svoboda, Institute of Experimental Biology and Genetics, Prague, Czechoslovakia. The cultures were grown in Eagle's minimal essential medium obtained in powdered form from Grand Island Biological Company, Grand Island, NY. The medium was supplemented with 10% tryptose phosphate broth (Difco, Detroit, Mi) and 10% unheated calf serum (KC Biological, Inc., Lenexa, Ka). The cultures were grown at 37 C in disposable 75 sq cm plastic flasks (Falcon Plastics, Oxnard, Ca) in either a closed system or an open system utilizing a humidified atmosphere of 3% CO_2-97% air. In the closed system, cells were normally planted in medium containing Hanks' salt base followed by a complete medium change 24 hours later utilizing medium containing Earle's salt base for increased buffering capacity. When cells were cultivated in the CO_2 incubator, medium containing Earle's base with the $NaHCO_3$ concentration reduced to 1.1 gm/liter was employed. All experiments which measured cellular growth, nucleic acid synthesis, nucleotide formation, or ara-A metabolism were performed at 37 C in the CO_2 incubator. Cultures were subcultured (1:4) at least every three days using 0.25% trypsin in in HBS.[30]

Total cell counts were made either in a hemocytometer or with a Model F Coulter Counter equipped with a 100 μm orifice. Viable counts were determined by means of trypan blue dye exclusion.

To increase the likelihood of detecting bacterial and/or mixed bacterial and mycoplasmal contamination, antibiotics were never included in any tissue culture media.[31] All cell lines were screened periodically (tests performed by Microbiological Associates, Bethesda, Md) and were found to be mycoplasma free.

CELLULAR GROWTH RATE AND LABELING OF RNA AND DNA.—B-mix K-44/6 cells were planted in growth medium at 1.5 × 10^6 cells/75 sq cm flask or 0.75 × 10^6 cells/25 sq cm flask. After 20–24 hours, cells were in early log phase and the medium was decanted and fresh Earle's salt base growth medium containing drug was added. Medium which contained ara-A was prepared immediately before use to minimize the degradation of the drug by adenosine deaminase present in calf serum.[29] When total nucleic acid synthesis was measured, sufficient [^{3}H]- or [^{14}C]thymidine or [^{3}H]uridine was added to a final concentration of 0.05–1.0 μCi/ml. In pulse label experiments, the isotope was added at the same final concentration and allowed to remain in contact with the cells for 30 minutes. The radioactive medium then was decanted, the cell monolayer washed twice with HBS and the cells harvested by means of 0.25% trypsin in HBS or with 0.25 m*M* EDTA (pH 8.0) plus 0.5% calf serum in HBS. Viable cells were enumerated as described above.

In order to determine the extent of labeled thymidine incorporation, 100 μl aliquots of cells were spotted on small numbered squares of Whatman No. 1 filter paper. After drying, the squares of filter paper were washed three times with ice-cold 5% TCA, twice with 95% ethanol, and once with anhydrous diethyl ether. The dried squares of filter paper were immersed in 15 ml of 0.3% PPO in toluene and radioactivity determined as described below. Alternatively, cells were precipitated with ice-cold 0.5 N $HClO_4$ and the acid-insoluble fraction washed by three resuspensions in 0.5 N $HClO_4$. Resulting pellets were dissolved in the toluene-Scintisol solution described below. The results of both procedures correlated well.

Incorporation of [^{3}H]uridine into RNA was measured by dissolving washed, acid-insoluble, cell pellets in 0.3 N KOH and hydrolyzing for 18 hours at 37 C. Following precipitation with $HClO_4$ and neutralization with KOH/$KHCO_3$, aliquots of the resulting supernatant solutions were added to a mixture of 0.3% PPO in toluene containing 10% Scintisol (Isolab, Akron, Oh). Samples were counted in a Beckman Model LS-250 or a Packard Model 3320 liquid scintillation spectrometer. Dpm were calculated for each isotope by the external standard method.

LABELING OF THYMIDINE NUCLEOTIDES.—B-mix K-44/6 cells were planted in disposable 100 × 20 mm petri dishes with 10 ml of growth medium at a concentration of 1.5 × 10^6 cells/dish. After 20–22 hours, cells were in early log phase and 1.0 ml of ara-A in growth medium was added to a final concentration of 120 μM. At selected times [^{3}H]thymidine was added to duplicate cultures in 0.2 ml of growth medium at a final concentration of 0.2 μCi/ml. Incubation at 37 C was continued for 0.5 hour, then radioactive medium was aspirated, cell sheets were rinsed twice with medium 199 without phenol red (Industrial Biological Laboratories, Rockville, Md), 2.0 ml of ice-cold 0.01 N acetic acid were added, and the cells scraped from the surface with a rubber policeman. (This method was used to remove the cell

sheet because separate experiments established that up to 40% of labeled intracellular nucleotides leaked from the cells when cell sheets were removed with trypsin or EDTA.) Cells were transferred to a conical tube, [^{14}C]ATP was added as an internal standard, and nucleotides extracted using the perchlorate procedure of Bagnara and Finch.[32] Acid insoluble material was washed free of soluble radioactivity by repeated resuspension and pelleting in ice-cold TCA. The insoluble material was dissolved in 0.2 ml of toluene (Packard Instrument Co., Downers Grove, Il), neutralized with acetic acid and dpm determined as indicated above. DNA was assayed by a diphenylamine procedure.[33]

Thymidine nucleotides were determined by thin-layer chromatography. KOH-neutralized perchlorate supernatants were concentrated by lyophilization and dried material was resuspended in 200 μl of water. Residual, insoluble perchlorate was removed by centrifugation. Aliquots of the supernatant fluids and a solution containing ATP, TTP, TDP, and TMP as reference compounds were spotted on PEI-cellulose thin-layer plates (Brinkman Instruments, Des Plaines, Il) and developed with water to remove residual thymidine. The plates then were chromatographed in the second dimension in 2 N formic acid - 2 M LiCl (1:1, v/v) according to Randerath and Randerath[34] to separate the nucleotides. Examination of developed plates under UV light (254 nm) revealed the reference compounds which appeared as dark spots. Radioactive metabolites were detected by cutting 0.5 or 1.0-cm wide sections of the plate into counting vials, extracting with 0.1 N HCl in methanol, adding 13 ml of 0.3% PPO in toluene solution and counting as indicated above. These procedures quantitatively recovered and detected all labeled compounds spotted on the plates. Coincidence of radioactive and reference compounds were taken as indicating identity.

Results

EFFECT OF ARA-A ON CELL POPULATION DOUBLING TIME.—Selected concentrations of ara-A were added to early log phase cultures of B-mix K-44/6 cells propagated in medium containing either horse serum or calf serum. The former medium gave a culture totally free of adenosine deaminase activity whereas the latter provided sufficient enzyme to degrade ara-A with a half-life of approximately 14 hours.[25] Monolayer cultures of B-mix cells normally exhibited a population doubling time of 19 hours when cultured in Eagle's minimum essential medium supplemented with horse serum and 14 hours when cultured in medium supplemented with calf serum. In the presence of 37 μM ara-A cells grown in the adenosine deaminase-free environment (horse serum supplement) underwent only 0.25 population doublings in 28 hours. With 150 μM ara-A there was no increase in viable cells (Fig 1A). In contrast, addition of 37 to 225 μM concentrations of ara-A to cells grown in medium containing adenosine deaminase (calf serum supplement) resulted in a cessation of mitosis for periods of 5 to 30 hours respectively. Following this quiescent period growth resumed at the original rate (Fig 1B). At the highest drug concentration tested (600 μM), cells did not resume mitosis and the number of viable cells decreased.

In order to determine if the administration of 600 μM ara-A resulted in a reversible inhibition of cell growth, ara-A was introduced to early log phase cells and then rinsed from the cultures at various times after addition of drug (Fig 2). When ara-A was removed 11 hours after addition, the cells rapidly resumed mitosis and divided with a normal doubling time. If drug removal was delayed until 35 hours, a further loss of viability was averted. When ara-A was removed 48 hours after addition virtually no recovery was noted. Cells rescued from ara-A inhibition did not reach the maximum cell density seen in sham-treated cultures.

EFFECT OF ARA-A ON DNA SYNTHESIS.—The effect of ara-A upon the synthesis of total cellular DNA was studied by the addition of selected amounts of drug and labeled thymidine to early log phase cells. Incorporation of labeled thymidine into TCA precipitable material decreased by 64%, 92% and 98% respectively after a 10- hour exposure to 37, 150 and 600 μM concentrations of ara-A (Fig 3). The effect of ara-A deamination on the rate of DNA synthesis was examined by pulse labeling cells grown in calf serum- or horse serum-supplemented medium. Ara-A treatment of cells cultured in adenosine deaminase-containing medium resulted in an initially rapid decrease in the incorporation rate of [^{3}H]thymidine. At 4 to 5 hours, a minimum rate was reached after which the incorporation rate recovered slightly and then remained relatively constant for 8 to 20 hours (Fig 4A). In the presence of 450 μM ara-A, there was no recovery in the rate of incorporation. When the experiment was repeated using medium devoid of adenosine deaminase,

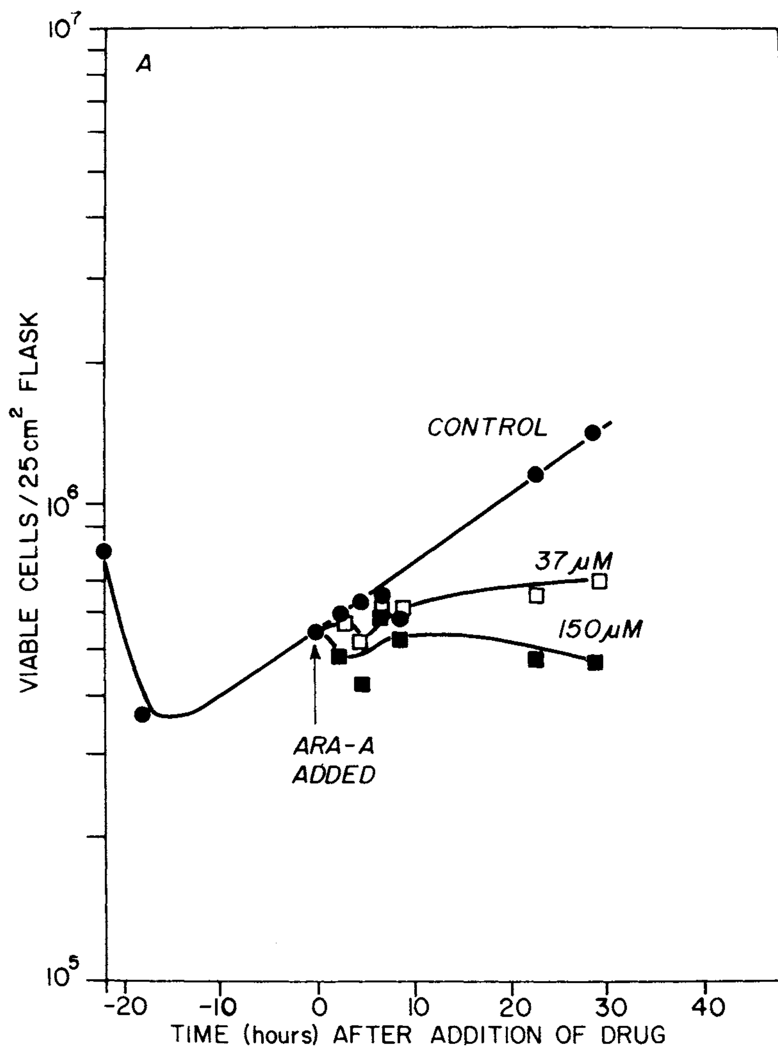

FIG 1.—Effect of ara-A on the growth of B-mix K-44/6 cells cultured in horse serum-supplemented medium (panel A) or in calf serum-supplemented medium (panel B). Drugs were added to identical monolayer cultures and following incubation duplicate flasks were harvested and cells enumerated at the times indicated. No drug (●), 37 μM (□), 150 μM (■), 225 μM (△) and 600 μM (×).

the rate declined during nearly all the time periods examined without any recovery to a constant rate of labeled thymidine incorporation (Fig 4B).

In order to learn whether the ara-A-induced decrease in incorporation of labeled thymidine actually represented decreased DNA synthesis or merely reflected a decreased labeling of DNA precursors, the effect of ara-A on the labeling of thymidine nucleotides was examined. Acid soluble nucleotides were extracted and characterized chromatographically following a 0.5-hour exposure of cells to [^{3}H]thymidine. Data listed in the Table show that during the time [^{3}H]thymidine incorporation into DNA was decreasing—and then recovering—there were no corresponding changes in the labeling of thymidine nucleotides. We conclude, therefore, that the observed decrease in labeled thymidine incorporation arose from a block-

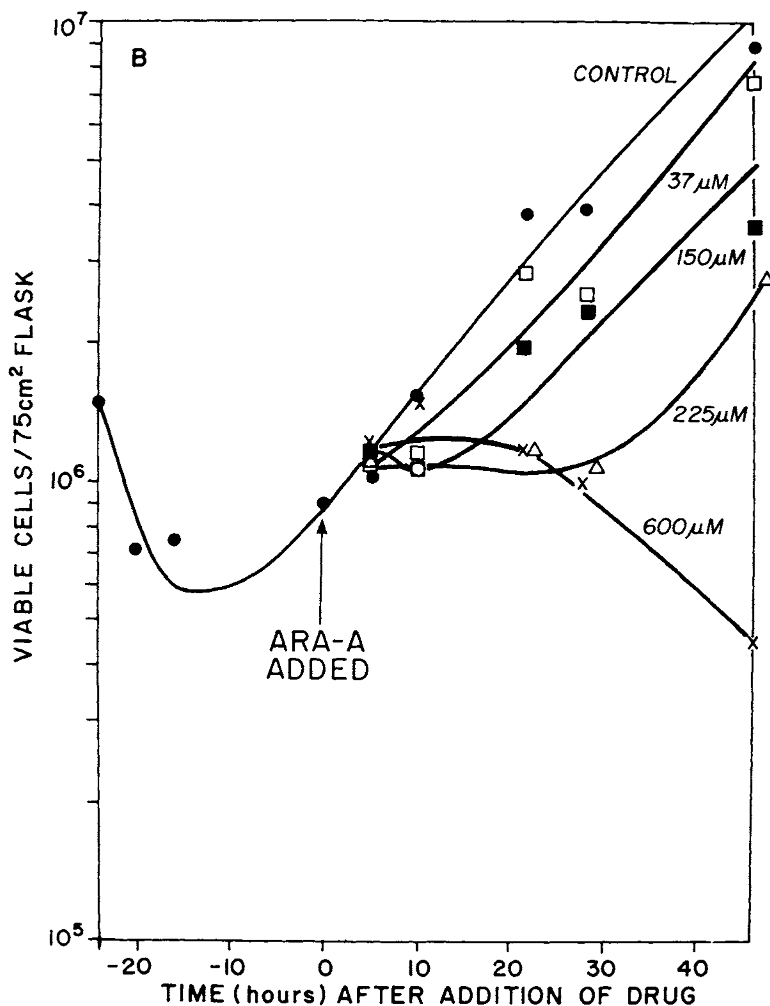

FIG 1.—Continued.

age of DNA synthesis by ara-A and not from an inhibition of thymidine uptake or phosphorylation.

The effect of ara-A on DNA synthesis in both continuous and pulse-labeled experiments is summarized in Fig 5. Inhibition following a 4- to 5-hour exposure to the drug was chosen to minimize the recovery of DNA synthesis in the deaminase-containing cultures. Even though the drug was degraded only to a minor extent (approximately 30%) during this time, the effect of deamination was apparent; ara-A was most active in the medium devoid of adenosine deaminase activity. In this medium, a 5.4 μM concentration of ara-A was required to inhibit DNA synthesis by 50% compared to a 9.5 μM concentration required in the presence of the enzyme.

EFFECT OF ARA-A ON RNA SYNTHESIS.—RNA synthesis in B-mix cells was measured by the incorporation of [^{3}H]uridine into acid precipitable material. Label incorporated into DNA was eliminated by KOH hydrolysis of RNA, precipitation of DNA and assaying the resulting supernatants for label. No effect of ara-A on RNA synthesis was apparent except at the concentration (600 μM) sufficient to cause cell death (Fig 6).

Discussion

We began the present work as background for studies on the mode of action of ara-A. When it became evident that the B-mix K-

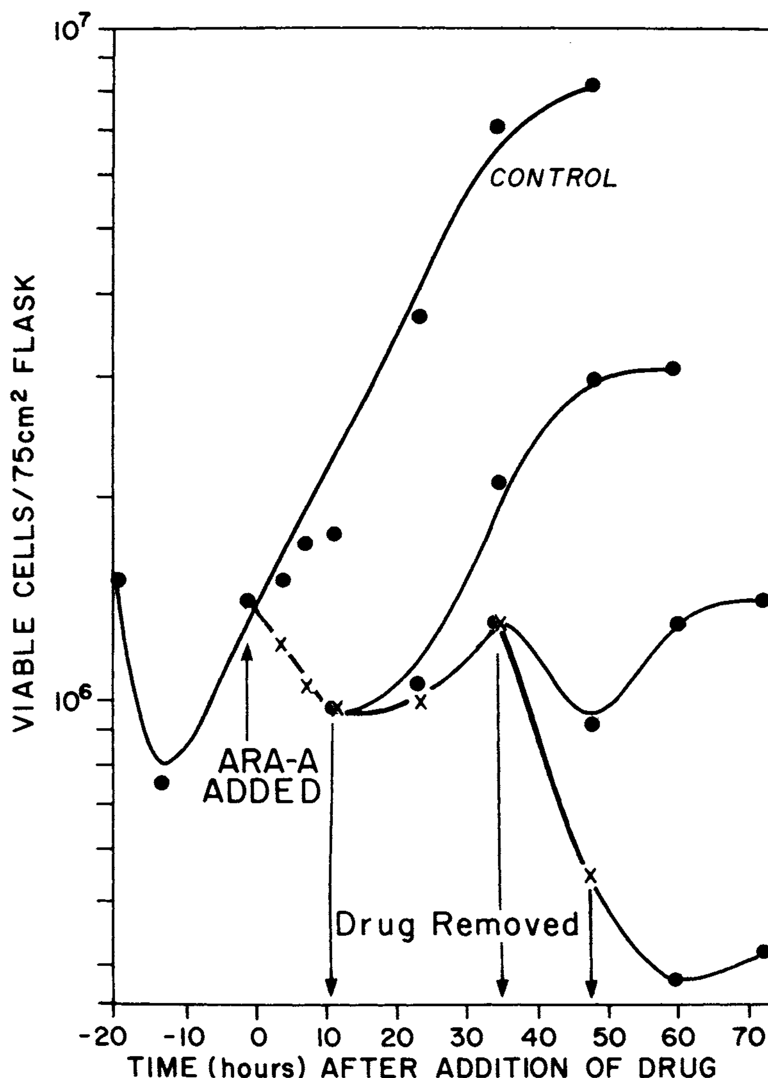

FIG. 2.—Influence of ara-A removal on the growth of B-mix K-44/6 cells. Drug (600 μM) was added to identical monolayer cultures grown in calf serum-supplemented medium and subsequently removed. At the times indicated, duplicate cultures were harvested and cells enumerated. Cell growth in the absence (●) and presence (×) of drug.

44/6 cell line was devoid of adenosine deaminase activity, the study was expanded to permit a direct comparison of the antiproliferative effects of the nucleoside under conditions where ara-A was, and was not, converted to ara-H.

Cells grown in medium with adenosine deaminase activity were inhibited initially but escaped inhibition within 5 to 20 hours depending on drug concentration (Fig 1B). In separate experiments (Fig 2), it was observed that the growth-inhibitory effects of ara-A also could be relieved by removing the drug-containing culture medium. Following removal of 600 μM ara-A, growth resumed within 10 to 15 hours. This is approximately the amount of time that would be needed for the cells to degrade intracellular arabinosyl nucleotides and to complete the S and G_2 phases of the cell cycle. Doering et al.[16] also found that L cells resumed multiplication 9 hours after removal of 200 μM ara-A suggesting that exposure to ara-A did not damage cells irrevocably. The re-

versibility of ara-A activity together with the low activity of ara-H strongly indicate that the resumption of cell growth in calf serum-supplemented medium resulted from the conversion of ara-A to ara-H. Results from experiments by Ortiz et al.[28] and by Plunkett and Cohen[22] seem to fit this pattern as well. These investigators found that the number of viable L cells (which contained adenosine deaminase) declined during the first 10- to 15-hour exposure to ara-A; subsequently the surviving cells resumed multiplication. The effect of ara-A on cell viability in other cell lines also reflects the enzymatic degradation of the drug.[35,36] In contrast, Leung et al.[37] observed that ara-H was nearly as lethal as ara-A to a polyauxotrophic strain of *Escherichia coli.* Thus, in *E coli,* conversion of ara-A to ara-H by cellular adenosine deaminase did not produce a less effective inhibitor and no resumption of cell growth occurred.

In mammalian cells, a lack of adenosine deaminase or its inhibition greatly increases the cytotoxicity of ara-A. In the present work, cellular growth was strongly inhibited by 37 μM ara-A and completely blocked by a 150 μM concentration when cells were propagated in an adenosine deaminase-free environment (Fig 1A). Recent cloning efficiency experiments by Plunkett and Cohen[22] and by Cass and Au-Yeung[23] also clearly established that exposure of cells to ara-A plus an adenosine deaminase inhibitor for periods greater than the population doubling time lead to nearly complete loss of cell viability.

The effect of ara-A on DNA synthesis paralleled its effect on cellular growth. The inhibition of DNA synthesis in B-mix K-44/6 cells cultured in adenosine deaminase-contain-

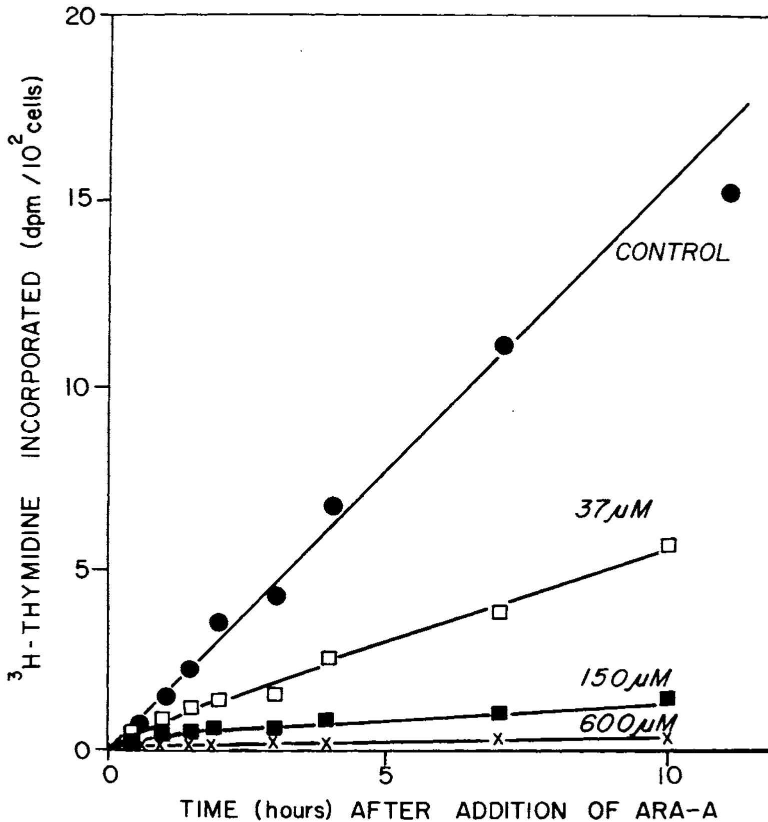

Fig 3.—Influence of ara-A on labeled thymidine incorporation. Selected concentrations of drug and [^{3}H]thymidine (1 μCi/ml, 15 Ci/mmole) were added to identical early log phase monolayer cultures 22 hours after planting in calf serum-containing medium. Duplicate flasks were harvested, precipitated and counted at the times indicated.

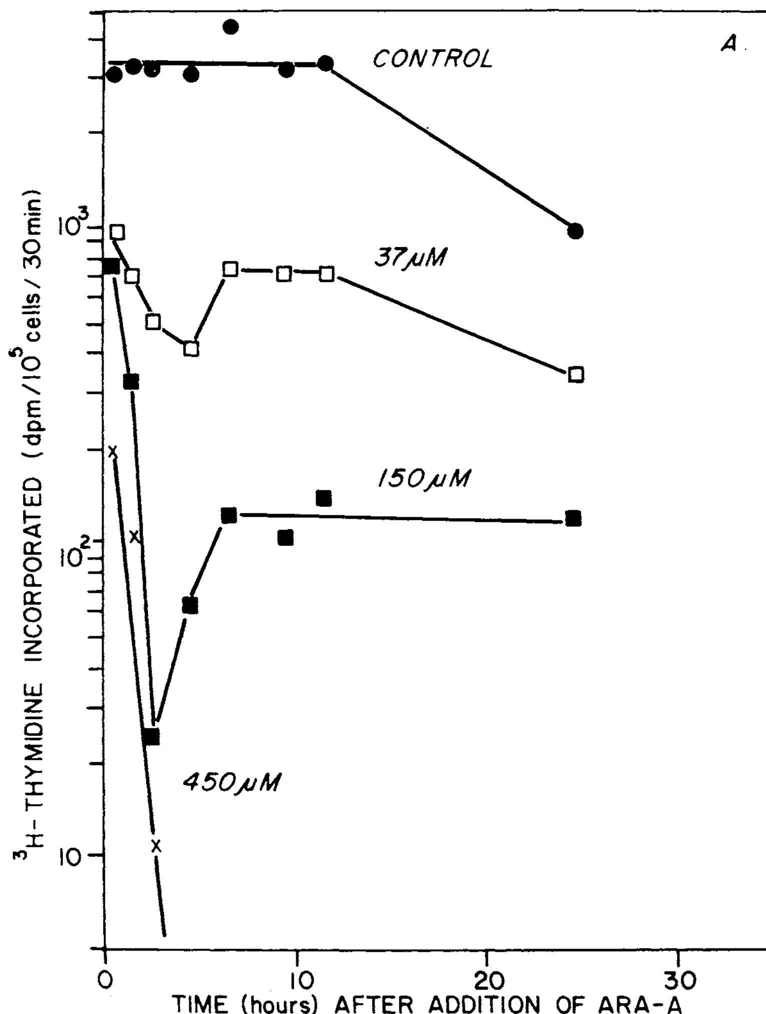

FIG 4.—Effect of ara-A on the rate of labeled thymidine incorporation by cells grown in calf serum-containing medium (panel A) or in horse serum-containing medium (panel B). Selected concentrations of ara-A were added to identical early log phase monolayer cultures 22 hours after planting. At the times indicated labeled thymidine (1 μCi/ml, 15 Ci/mmole panel A; 2 μCi/ml, 45 Ci/mmole panel B) was added 0.5 hour before duplicate flasks were harvested.

ing medium was approximately one-half that observed in cells cultured in the deaminase-free medium, *viz.* 10 *μM* was required to obtain 50% inhibition when measured 4 to 5 hours after drug addition (Fig 5). However, these observations were made at time points < 1/3 the biological half-life of ara-A in the medium. The activity of ara-A at times twice as long as the biological half-life, best measured by pulse exposures to labeled thymidine (Fig 4), revealed a considerably more potent inhibitory effect for the drug in adenosine deaminase-free medium. For example, in medium which deaminated the drug, inhibition of DNA synthesis 20 hours after drug addition was 78% and 96% at ara-A concentrations of 37 and 150 *μM*, respectively. In deaminase-free medium, inhibitions of 94% and 99.6% were noted at the same time period. In addition, the recovery in the rate of DNA synthesis noted in cells grown in the deaminase-containing medium was almost abolished in cells grown in deaminase-free me-

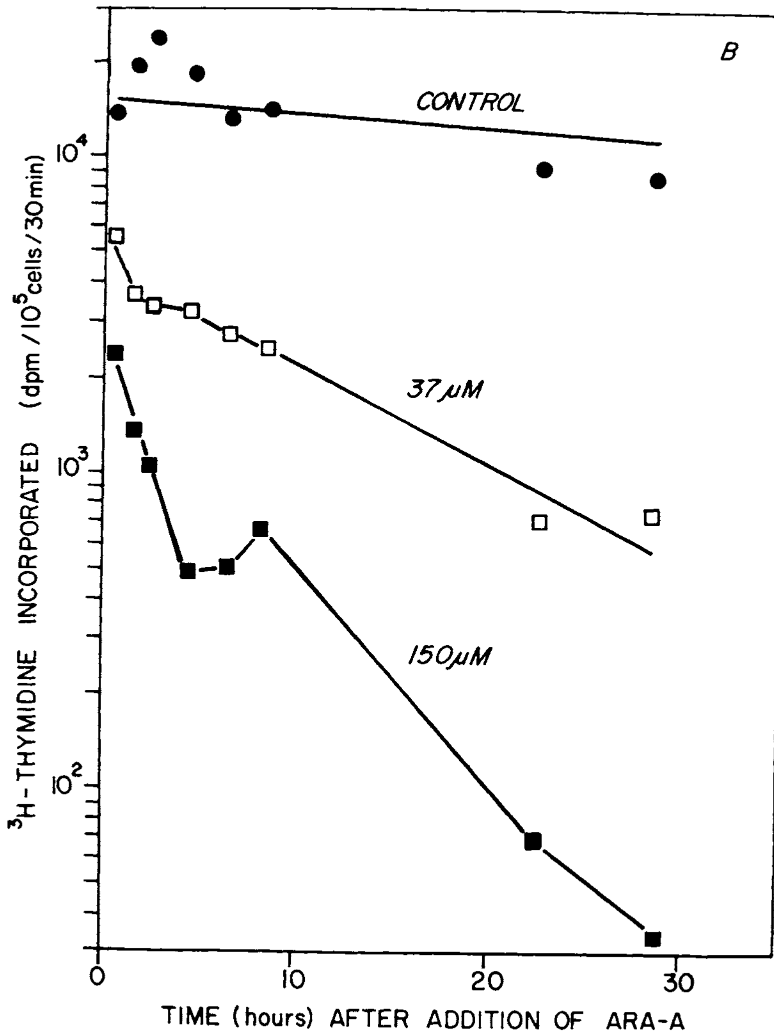

FIG 4—Continued.

dium (Fig 4). In unpublished experiments, we have observed a more nearly complete recovery in the rate of DNA synthesis following exposure of KB cells (an established line of human epidermoid carcinoma cells) to ara-A for 6 to 12 hours. Ara-A has a half-life of 1 to 2 hours in monolayer cultures of KB cells; thus the more complete recovery in the rate of DNA synthesis may be related to the more rapid inactivation of the drug. LePage,[17] working with mice bearing Ehrlich ascites carcinoma and Cass and Au-Yeung,[23] using L1210 cells in vitro also observed a marked inhibition of cellular DNA synthesis 15 to 30 minutes after administration of ara-A. Subsequently, DNA synthesis recovered and surpassed control values by 12 to 24 hours after dosing. No recovery was observed when the adenosine deaminase inhibitor covidarabine was added.[23] Recovery of DNA synthesis following inhibition by ara-C also has been reported.[38,39]

The activity of ara-A against DNA synthesis in B-mix K-44/6 cells cultured in adenosine deaminase-containing medium is approximately the same as that noted in vitro by other investigators. For example, Grindey and Nichol[40] found that a 13 to 31 μM concentration of ara-A was required for a 50% inhibition. Doering et al.[16] reported that a 200 μM concentration almost totally inhibited DNA synthesis. Less potent effects of ara-A have been reported by Brink and LePage[41] who observed

TABLE

EFFECT OF ARA-A ON LABELING OF INTRACELLULAR THYMIDINE NUCLEOTIDES IN B-MIX K-44/6 CELLS

Time (hr) after addition of 120 μM ara-A	Incorporation of [³H] Labeled Thymidine (dpm/μg DNA)*				
	TMP	TDP	TTP	DNA	DNA (% Control)
Control	26†	156†	2330†	1700	100
0.5	29	243	2740	405	24
1.5	47	276	2430	252	15
2.5	23	218	2840	137	8.1
4.5	63	208	1650	113	6.6
6.5	13	124	1520	132	7.8
8.5	30	179	2350	230	14
10.5	30	145	1710	316	19
24.5	22	128	1710	411	24

Note: Duplicate monolayer cultures of cells logarithmically growing in calf serum-supplemented medium were exposed to 0.2 μCi/ml of [³H]thymidine for 0.5 hour at selected times. Cells were harvested and radioactive, acid-soluble, nucleotides were characterized by liquid scintillation counting of sectioned thin-layer chromatograms. Radioactivity incorporated into DNA was determined in the corresponding acid-insoluble precipitates.

* Nucleotide dpm also were volume corrected by the addition of an internal standard ([¹⁴C]ATP) to acid-soluble fractions.

† Average values from cultures without drug. Cultures were incubated for 0 to 6 hours after sham treatment and then exposed to [³H]thymidine for 0.5 hour. Prior experiments had established that such labeling of thymidine nucleotides was constant over a 24-hour period in logarithmically growing cells.

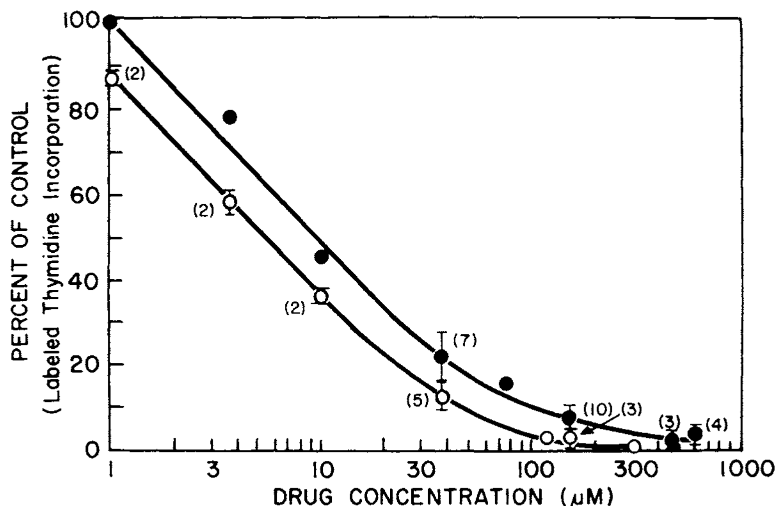

FIG 5.—Dose-response relationships illustrating the inhibition of labeled thymidine incorporation by ara-A. Data from all experiments (continuous and pulse labeled) are expressed as the average activity (± standard deviation) remaining after a 4- to 5-hour exposure of cells to the drug. Numbers in parentheses give the number of replicate experiments at each drug level. Duplicate cultures were used in all experiments. Activity of ara-A cells cultured with horse serum-supplemented medium (O) or calf serum-supplemented medium (●).

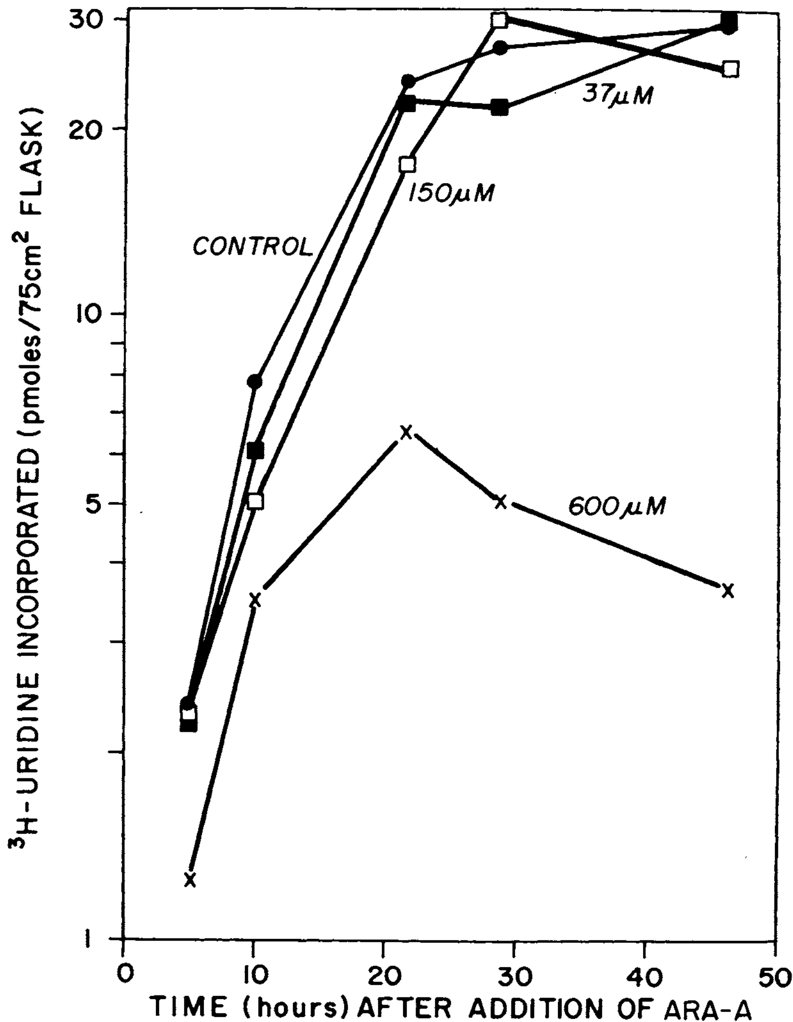

FIG 6.—Influence of ara-A on labeled uridine incorporation by cells grown in calf serum-containing medium. Selected concentrations of drug and [^{3}H]uridine (0.2 μCi/ml, 26 Ci/mmole) were added to identical early log phase monolayer cultures 22 hours after planting and duplicate flasks were harvested at the times indicated.

approximately 80% inhibition of DNA synthesis at 750 *μM* ara-A and by Hughes and Kimball[42] who noted an approximate 70% reduction with 1 m*M* ara-A. The lower activity in the latter two studies might have arisen through the use of much heavier cell suspensions (3.5×10^7 cells/ml) than used in our studies ($1–2 \times 10^6$ cells/25 sq cm bottle) and the resultant exposure of ara-A to high levels of adenosine deaminase. In previous work with KB cells,[13] which degrade ara-A with a shorter half-life (1 to 2 hours) than in the present study, we found that 67 *μM* ara-A was needed to inhibit cellular DNA synthesis by 50% when adenosine deaminase was not inhibited and 8 *μM* when it was inhibited by coformycin. The latter figure agrees well with the 5 *μM* concentration required in the current study using adenosine deaminase-free cultures. The lower potency of ara-A reported in our earlier work with B-mix cells (80% inhibition at 500 *μM* ara-A)[43] probably was observed because labeled thymidine incorporation was measured after a 22-hour exposure to the drug thereby permitting significant conversion to ara-H and consequent recovery in DNA synthesis. However,

both our prior and our current results differ from those of Bennett et al.[44] who observed that concentrations of ara-A up to 375 μM did not inhibit DNA synthesis in HEp-2 cells. Even assuming that ara-A was totally converted to ara-H by HEp-2 cells prior to the 9-hour labeling period employed, some inhibition would be expected. However, a change in the labeling of thymidine nucleotides during the HEp-2 cell experiments[44] may be sufficient to explain the apparent inability of the drug to inhibit DNA synthesis.

To our knowledge, all other investigators who utilized incorporation of labeled thymidine as a measure of DNA synthesis did so without eliminating the possibility that the apparent inhibition of DNA synthesis by ara-A might reflect a decrease in the phosphorylation of the labeled precursor. The observation that [^{3}H]-thymidine phosphorylation was not affected by 120 μM ara-A (Table) establishes that inhibition of labeled thymidine incorporation by ara-A does reflect inhibition of DNA synthesis. Although ara-C decreases thymidylate synthetase activity as a secondary drug effect,[39] like ara-A it does not affect the uptake or phosphorylation of thymidine at concentrations needed to inhibit DNA synthesis. Graham and Whitmore[45] found no change in the labeling of thymidine nucleotides during a $> 99\%$ inhibition of DNA synthesis by ara-C. Roller et al.[46] also noted that concentrations of ara-C as high as 411 μM did not influence thymidine uptake into acid-soluble material. Chou et al.[38] found that ara-C did not affect labeled pools of cytidine and its nucleotides during inhibition of DNA synthesis. In contrast, Bennett et al.[44] observed a threefold increase in the labeling of thymidine nucleotides in the presence of 375 μM ara-A. Although differences between Bennett's observations and ours might arise from the use of different cell lines or from different times of exposure to labeled thymidine (9 hours versus 30 minutes in this study), we have no data which reconcile the dissimilarities.

Little or no effect of ara-A has been observed on RNA synthesis in *E coli*,[37] ascites tumor cells,[15,41] L cells[16] and HEp-2 cells.[44] The lack of effect of $\leq$ 150 μM ara-A on RNA synthesis in our studies is consistent with these observations. Inhibition of RNA synthesis was observed at 600 μM ara-A but this concentration was sufficient to cause cell death (Fig 1B). Inhibition of RNA synthesis also has been noted following exposure of MDBK cells to high levels (560 μM) of drug.[47]

Conclusions

The effect of ara-A on cellular growth, DNA synthesis, and RNA synthesis was measured in an established cell line (B-mix K-44/6) devoid of adenosine deaminase activity. Cells adapted to growth in a medium supplemented with horse serum provided an environment totally lacking adenosine deaminase activity whereas cultivation of cells in a medium supplemented with calf serum provided a system capable of deaminating ara-A to ara-H (half-life = 14 hours). Under deaminase-free conditions early log phase cells underwent 1.5 population doublings during 28 hours compared with 0.25 doublings in the presence of 37 μM ara-A. When cells were grown in medium supplemented with calf serum the addition of 37 to 225 μM ara-A resulted in a cessation of mitosis for periods of 5 to 30 hours respectively. Following this quiescent period growth resumed at the original rate. With 600 μM ara-A mitosis was reversibly inhibited up to 35 hours after drug addition.

The effects of ara-A on RNA and DNA synthesis were monitored by continuously or pulse labeling B-mix K-44/6 cells with [^{3}H]-uridine or [^{3}H]thymidine. Ara-A did not influence RNA synthesis as judged by labeled uridine incorporation. Under deaminase-free conditions, 5.4 μM ara-A inhibited labeled thymidine incorporation by 50%. In the presence of the enzyme, approximately twice the ara-A concentration was required for the same inhibition; furthermore, the initial inhibition was followed by a partial recovery in the rate of thymidine incorporation. Examination of thymidine nucleotide pools during ara-A treatment revealed no changes in the labeling of dTMP, dTDP, and dTTP. Thus inhibition of [^{3}H]thymidine incorporation by ara-A accurately reflected inhibition of DNA synthesis.

We conclude that, in spite of an initial inhibition of DNA synthesis and mitosis by ara-A, B-mix K-44/6 cells recover from the inhibitory effects if the drug is removed either by a change in the culture medium or by metabolism to ara-H.

We thank Mrs. Sandra H. Smith for her excellent technical assistance. Stimulating discussions with Drs. Theodore R. Oegema and Pauline M. Schwartz are appreciatively acknowledged.

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