Effect of 1-β-D-Arabinofuranosylcytosine on DNA Synthesis

II. In Rabbit Kidney Cells Infected with Herpes Viruses

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SUMMARY

1-β-D-Arabinofuranosylcytosine (Ara-C) inhibits the synthesis of DNA in noninfected rabbit kidney cells to a greater degree than in cells infected with viruses of the herpes group (herpes simplex and pseudorabies viruses). Virus multiplication is inhibited by the drug to the same extent as DNA synthesis, demonstrating that Ara-C interferes with the infective process by inhibiting the synthesis of DNA only.

The relative resistance of infected cells to the action of the drug is probably due to the lower ability of these cells as compared to noninfected cells to phosphorylate Ara-C. The low level of phosphorylation of the drug in the infected cells is correlated with a decrease in the activity of deoxycytidine kinase *in vivo*. Furthermore, the drug does not induce in the infected cells an increase in the level of activity of deoxycytidine kinase, an effect it does produce in noninfected cells. It is concluded that Ara-C is not a specific antiviral agent.

INTRODUCTION

The drug Ara-C[1] interferes with the synthesis of DNA in a variety of mammalian cells (1–4) and has been studied for its possible antiviral activity (5–7, 20). It has been reported that Ara-C is effective in the control of herpes keratitis, although it seems to be slower than IUdR in clearing the initial infection (8, 9) and is considerably more toxic than IUdR to the host cells (10).

[1] Abbreviations: Ara-C, 1-β-D-arabinofuranosylcytosine; Ara-CMP, Ara-C monophosphate; ATP, adenosine triphosphate; dATP, deoxyadenosine triphosphate; CR, cytidine; CdR, deoxycytidine; CDP, cytidine diphosphate; dCDP, deoxycytidine diphosphate; dCMP, deoxycytidine monophosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; TdR, thymidine; dTMP, thymidine monophosphate; dTTP, thymidine triphosphate; UdR, uridine; dUMP, deoxyuridine monophosphate; IUdR, 5-iodo-2'-deoxyuridine; PCA, perchloric acid; RK, rabbit kidney.

We have shown recently that IUdR may be a "selective" antiviral agent: conditions can be created to permit greater incorporation of IUdR into the DNA of infected cells than into the DNA of noninfected cells (11, 12). In the present paper, experiments are presented that were designed to determine whether Ara-C, in spite of its relatively high toxicity to noninfected cells, interferes more drastically with the metabolism of infected cells than of noninfected cells. The experiments will show that, in contrast to IUdR, Ara-C is not a "selective" antiviral drug. Preliminary accounts of some of these experiments have been reported previously (11, 13).

MATERIALS AND METHODS

Cells and Media

The cells and media used for these experiments were the same as those described in the preceding paper (14).

Viruses

The properties and assay of pseudorabies virus and herpes simplex virus have been described previously (15).

Enzyme Assays

The preparation of cell extracts has been described previously (16).

CdR kinase was assayed as described in the preceding paper (14).

DNA polymerase. One-tenth milliliter of cell extract (approximately 0.5 μg protein) was added to 0.15 ml of reaction mixture containing 60 μg of heat-denatured calf thymus DNA, 2.5 μmoles of ATP, 25 μmoles of Tris pH 7.5, 5 μmoles of $MgCl_2$, 50 mμmoles each of dTTP and dGTP, 250 mμmoles of dCMP, 6 mμmoles of ^{3}H-dATP (specific activity for infected cell extract, 0.21 μC/mμmole; specific activity for noninfected cell extract, 1.05 μC/mμmole), as well as the nucleotides listed in Table 1. (Under the conditions used, the inclusion of Ara-CMP in the incubation mix did not significantly affect the formation of dCTP from dCMP. This was probably so because approximately 15 times more dCMP than Ara-CMP was present in the incubation mix. dCMP was used instead of dCTP as one of the precursors for the DNA to be synthesized because dCTP may prevent the phosphorylation of Ara-CMP and consequently may prevent the inhibitory effect of the latter on DNA polymerase.) The samples were incubated for 1 hr at 37° and were precipitated with cold PCA. After extensive washing in cold PCA, the radioactivity incorporated into DNA was determined in a Packard spectrometer.

The estimation of the reduction of CDP to dCDP, of the phosphorylation of CdR and of Ara-C *in vivo*, as well as the incorporation of radio chemicals into DNA, were performed as described in the preceding paper (14).

The activity of dCMP deaminase *in vivo* in infected cells was tested by incubating these cells with ^{3}H-CdR. The acid-soluble pool was obtained, as described previously (14), and was heated in PCA (1 *N*) to convert the deoxyribonucleotide di- and triphosphates to the corresponding monophosphates. The deoxyribonucleotide monophosphates present in DNA were obtained as described previously (17). dCMP was separated from dUMP (and dTMP) on Dowex 1-formate columns, as described previously (17).

To test for the activity *in vivo* of CdR phosphorylase, the infected cells were incubated with ^{3}H-CdR and the acid-soluble pool obtained as described above. CdR was separated from cytosine by the method described by Decker (18).

Ara-CTP was prepared enzymatically from Ara-CMP and separated from Ara-CMP on Dowex 1-HCl columns.

Chemicals. ^{3}H-CdR, ^{3}H-CR, ^{14}C-TdR, and ^{3}H-Ara-C were purchased from Schwarz BioResearch Inc. Ara-C was a gift from Wayne Magee, the Upjohn Company; Ara-CMP was a gift of William Wechter, the Upjohn Company.

RESULTS

Inhibition by Ara-C of DNA Synthesis in Infected and Noninfected RK Cells

The effect of various concentrations of Ara-C on the synthesis of DNA in noninfected cells and in cells infected with either herpes simplex virus or pseudorabies virus was tested. For this purpose, actively growing RK monolayer cultures were used, in which there is after infection with these viruses little change in the overall rate of DNA synthesis (19).

Figure 1 shows that DNA synthesis (as determined by the incorporation of ^{14}C-TdR into DNA) is much less affected by Ara-C in the infected cells than in the noninfected cells. Thus, a concentration of Ara-C (0.05 μg/ml) that inhibited the incorporation of ^{14}C-TdR into the DNA of noninfected cells by about 50% had no effect on its incorporation in pseudorabies virus-infected cells and only a slight effect on its incorporation in herpes simplex virus-infected cells (about 15%). Similar results were obtained when the incorporation of ^{3}H-CR or ^{3}H-adenine into DNA was tested.

Although it has been established in a variety of systems that Ara-C inhibits specifically the synthesis of DNA (20), it seemed possible, nevertheless, that in addi-

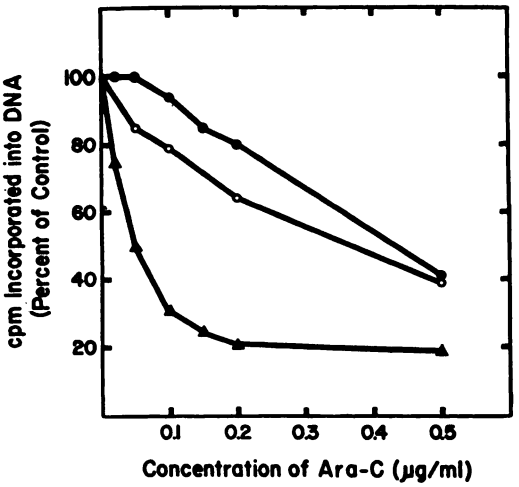

FIG. 1. *Effect of various concentrations of Ara-C on the incorporation of ^{3}H-TdR into the DNA of noninfected cells and of cells infected with pseudorabies or herpes simplex virus*

Actively growing cultures were infected with pseudorabies virus or herpes simplex virus (adsorbed multiplicity = 10). Mock-infected cultures served as controls. The cultures were incubated for 5 hr in EDS containing Ara-C. ^{14}C-TdR (0.04 μC/ml; specific activity, 31 mC/mmole) was then added to each culture. One hour later the cultures were harvested and the amount of radioactivity in the DNA was determined. Pseudorabies virus-infected cells (●); herpes simplex virus-infected cells (○); noninfected cells (▲).

tion to inhibiting viral DNA synthesis, the drug might also affect other aspects of virus replication. In the next experiment, we compared the effect of various concentrations of Ara-C on DNA synthesis and on virus production in virus-infected cells. Figure 2 shows that as the concentration of Ara-C was increased, the amounts of ^{14}C-TdR incorporated into DNA and of infectious virus produced were decreased to the same degree. These results indicate that the inhibition of virus synthesis by Ara-C is due primarily to the effect of the drug on DNA synthesis.

Mechanism of Relative Resistance of Infected Cells to Ara-C

Effect of Ara-C derivatives on DNA polymerases in extracts of infected and noninfected cells. In order to understand the basis for the greater resistance of infected than noninfected cells to Ara-C, it is essential to understand the mode of action of the drug on DNA synthesis. The experiments of Chu and Fischer (1) suggested that Ara-C may be effective in inhibiting DNA synthesis by interfering with the reduction of CDP to dCDP. This is not the case in RK cells (14). Ara-C may also inhibit DNA synthesis by interfering with the polymerization of deoxyribonucleotides as a result either of its incorporation into DNA (4, 21), or of its inhibition of DNA polymerase activity (20).[2] Infection with pseudorabies virus induces an increase in the level of activity of DNA polymerase

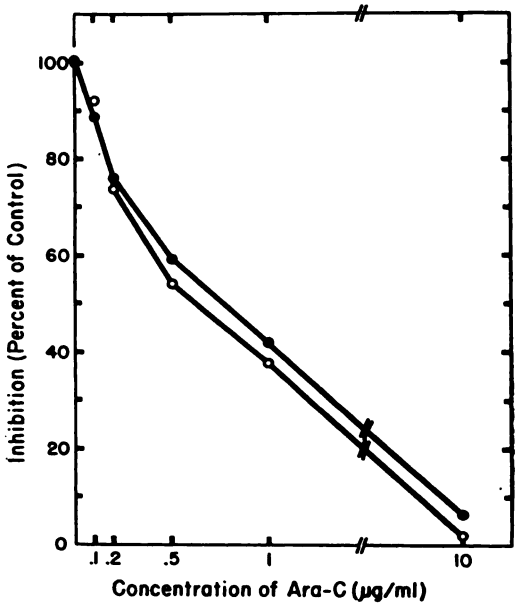

FIG. 2. *Correlation between the degree of inhibition by Ara-C of the synthesis of DNA and of infectious pseudorabies virus in infected RK cells*

Actively growing cultures were infected (adsorbed multiplicity = 2), were washed extensively to remove unadsorbed virus, and were incubated in EDS. Three hours after inoculation, the medium was changed to EDS containing TdR (0.1 μg/ml), various concentrations of Ara-C, and ^{14}C-TdR (0.04 μC/ml; specific activity, 31 mC/mmole). The cultures were harvested 12 hr after infection and the number of plaque-forming units produced by each culture, as well as the amount of ^{14}C-TdR incorporated into DNA, was determined. Incorporation of ^{14}C-TdR (○); infectious virus (●).

[2] J. J. Furth and S. S. Cohen, personal communication.

TABLE 1

Effect of Ara-CMP on DNA polymerase activity present in extracts of infected and noninfected cells

Extracts prepared from infected and noninfected cells were assayed for DNA polymerase activity in the presence of the indicated amounts of nucleotides, as described in Materials and Methods. The results are the average of duplicate samples. The maximum variation between the individual samples was 10%.

		Incorporation of dATP into DNA			
		Infected cell extract		Noninfected cell extract	
Nucleotide added	μg/sample	μμmoles/ sample	Inhibition (%)	μμmoles/ sample	Inhibition (%)
None	—	7.70	—	1.16	—
Ara-CMP	2	4.32	44	0.72	38
Ara-CMP	5	3.82	51	0.59	49
dCTP	50	7.93		1.31	
dCTP + Ara-CMP	50 5	7.76	2	1.37	0

(22). The relative resistance of infected cells to Ara-C as compared to noninfected cells may thus be due to the presence in the infected cells of a DNA polymerase that is less affected by Ara-C derivatives than the enzyme present in noninfected cells.

The effect of Ara-CMP on the polymerization of deoxyribonucleotides into DNA by extracts prepared from infected and noninfected cells was tested. The results of this experiment (Table 1) show that Ara-CMP inhibits DNA polymerase activity in both infected and noninfected cell extracts and that this inhibition is completely reversed by dCTP. Similar results were also obtained when Ara-CTP was used instead of Ara-CMP. (These experiments were carried out with crude extracts of cells containing a high level of activity both of dCMP kinase and of phosphatases, and there was presumably interconversion between Ara-CMP and Ara-CTP.) The activity of DNA polymerase in the infected cells was *not* more resistant to the inhibitory effect of Ara-CMP than the activity of the enzyme in the noninfected cells, and it is unlikely therefore that the induction of a different DNA polymerase in infected cells can be the basis for the relative resistance of the virus-infected cells to Ara-C. It is possible, however, that although the polymerases from both sources are equally sensitive to phosphorylated derivatives of Ara-C, the greater activity of the enzyme in infected cells than in noninfected cells may contribute to the greater resistance of the infected cells to the drug.

Phosphorylation of Ara-C by infected and noninfected cells. It has been shown by Chu and Fischer (21) that a mutant cell line resistant to Ara-C is unable to phosphorylate this drug and lacks CdR kinase, suggesting that Ara-C must be phosphorylated in order to affect DNA synthesis. We tested therefore whether the relative resistance of the virus-infected cells to Ara-C could reside in a decrease in the ability of these cells to phosphorylate it. This was done by comparing the amount of phosphorylated Ara-C which accumulates in the intracellular acid-soluble pools of infected and noninfected cells.

The results of this experiment (Table 2) show that the infected cells phosphorylated approximately four times less Ara-C than did the noninfected cells. (In other experiments, differences as great as tenfold have been observed.) Since presumably only the phosphorylated derivatives of the drug exert an inhibitory action on DNA synthesis, the relative resistance of infected cells may be ascribed to the low level of phosphorylation of Ara-C in these cells.

The low level of accumulation of phos-

TABLE 2
Phosphorylation of ^{3}H-Ara-C in infected and noninfected cells

Actively growing RK cells were infected (or mock infected) with pseudorabies virus (adsorbed multiplicity = 10). Four hours after infection, the cultures were incubated for 3 hr in EDS medium containing 0.8 μC ^{3}H-Ara-C/ml to which unlabeled Ara-C was added to give the concentration indicated. The amount of radioactivity associated with phosphorylated Ara-C in the intracellular pool was determined, and the amount of Ara-C phosphorylated by the cultures was calculated. The results are the average of duplicate samples. The maximum variation between the individual samples was 7%.

Concentration of Ara-C (μg/ml)	Ara-C phosphorylated	
	In infected cells	In noninfected cells
0.05	0.22[a]	0.97
0.1	0.49	1.97
0.2	1.28	3.90
0.5	2.41	9.29
2.0	7.47	33.10

[a] Micrograms phosphorylated per sample × 10^2.

phorylated derivatives of Ara-C in the infected cells could be due to the rapid deamination of Ara-CMP. That this is not the case, however, is shown by the following experiment: RK cells were infected with a high multiplicity of pseudorabies virus and incubated in EDS. Noninfected cells treated identically served as controls. Five hours after infection ^{3}H-CdR (5 μC/ml; specific activity, 625 μC/mmole) was added to the cultures, which were incubated further for 3 hours. The phosphorylated radioactive deoxyribonucleotides present in the acid-soluble pool and in the DNA were converted to deoxyribomononucleotides and were separated from each other, as described in Materials and Methods. (The deoxyribomononucleotides were examined because the cells contain a high level of activity of TdR kinase (22) and UdR, resulting from the deamination of CdR, would be rapidly phosphorylated.) The amount of radioactivity associated with each was determined. Both in the infected and in the noninfected cells at least 95% of the radioactivity was found in dCMP, indicating that the *in vivo* level of dCMP deaminase activity in RK cells is relatively low and remains low after infection of these cells with pseudorabies virus.

We also tested the level of activity *in vivo* of CdR phosphorylase. The experiment was performed as described above and the amount of ^{3}H-CdR present in the intracellular pool, as well as in the culture medium, was determined as described in Materials and Methods. Under the conditions used, no detectable phosphorolysis of CdR was found either in the infected or in the noninfected cells.

Since the level of activity *in vivo* both of dCMP deaminase and of CdR phosphorylase is low in the infected, as well as in the noninfected cells, the lower level of phosphorylated Ara-C present in the infected cells is probably due to a decrease in the ability of the cells to phosphorylate Ara-C.

We have reported previously that there is no difference in the level of CdR kinase activity in extracts prepared from infected and noninfected cells (22). However, despite the similar levels of activity of this enzyme *in vitro*, the ability of infected cells to phosphorylate CdR *in vivo* is consider-

TABLE 3
Phosphorylation of ^{3}H-CdR in infected and noninfected cells

Actively growing RK cells were infected (or mock-infected) with pseudorabies virus (multiplicity = 10) and were incubated in EDS for 4 hr. ^{3}H-CdR (5 μC/ml; specific activity, 625 mC/mmole) was added and 3 hr later the cells were harvested and the amount of ^{3}H-CdR which had been phosphorylated and was present in the acid-soluble pool and in DNA was determined. The results are the average of duplicate samples. The maximum variation between the individual samples was 13%.

Treatment	Phosphorylated ^{3}H-CdR present		
	Acid-soluble pool	DNA	Total
Infected	9,500[a]	17,410	26,910
Noninfected	36,300	54,200	90,500

[a] Cpm ^{3}H-dCMP/sample.

TABLE 4

Reduction of ^{3}H-CR in infected and noninfected cells

Actively growing RK cells were infected with pseudorabies virus (adsorbed multiplicity = 10) and were incubated in EDS. Four hours after infection, ^{3}H-CR (5 μC/ml; specific activity, 15 C/mmole) was added to the cultures. Three hours thereafter the cells were harvested and the amount of radioactivity present as CMP and dCMP in the acid-soluble pool, as well as dCMP in DNA, was determined.

Treatment	^{3}H-CMP in acid-soluble pool	^{3}H-dCMP in acid-soluble pool	^{3}H-dCMP in DNA	Total ^{3}H-CDP reduced
Infected	700[a]	20.0	18.1	38.1
Noninfected	500	4.8	7.7	12.5

[a] Cpm/sample $\times 10^{-3}$.

ably lower than that of noninfected cells (Table 3), even though these cells have approximately similar rates of DNA synthesis, as measured by ^{32}P- and ^{3}H-TdR incorporation into DNA. This could be due to the fact that the endogenous pool of dCTP is increased in the infected cells, reducing thereby the level of activity of CdR kinase by feedback inhibition.[2]

That the acid-soluble pool of endogenously formed CdR derivatives is indeed increased in infected cells is evident from Table 4, which shows that the amount of ^{3}H-CR reduced by these cells, as compared to the noninfected cells, is increased by a factor of about three. The relatively high level of dCMP (and dCTP) derived from CR present in the infected cells does not constitute proof that the decrease in the activity of CdR kinase *in vivo* is the result of a feedback inhibition exerted by dCTP. However, this seems to be a reasonable explanation of our results.

Effect of Ara-C on the induction of CdR kinase in pseudorabies virus-infected cells. Incubation of noninfected cells with Ara-C induces an increase in the level of activity of CdR kinase and probably increases the ability of the cells to phosphorylate the drug (14). We tested whether this was also the case in pseudorabies virus-infected cells.

Infected and noninfected cells were incubated with various concentrations of Ara-C. The cells were harvested, extracts were prepared, and the ability of these extracts to phosphorylate CdR was determined. Figure 3 shows that whereas incubation with Ara-C induced an increase in the level of CdR kinase activity in noninfected cells, it did not do so in infected cells.

The lower level of phosphorylation of Ara-C in infected cells than in noninfected cells can be attributed in part, therefore, to the fact that the increase in the level of

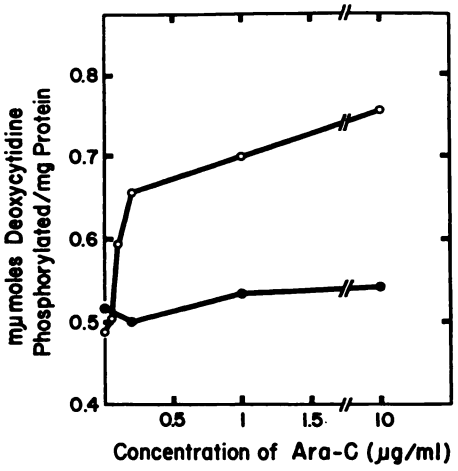

FIG. 3. *Effect of incubation with Ara-C on the induction of CdR kinase activity in infected and noninfected cultures*

Actively growing RK cultures were infected with pseudorabies virus (or mock-infected) (adsorbed multiplicity = 10) and were incubated for 4 hr in EDS containing various concentrations of Ara-C. The cultures were harvested, extracts were prepared, and the activity of CdR kinase was determined, as described in Materials and Methods. Infected cultures (●); noninfected cultures (○).

activity of CdR kinase, which occurs in noninfected cells incubated with Ara-C, does not occur in infected cells.

DISCUSSION

The experiments in this paper show that Ara-C is more effective in inhibiting DNA synthesis in noninfected cells than in infected cells. The relative resistance of infected cells to Ara-C is due to the relatively low level of phosphorylation of the drug by these cells and is related to a decrease after infection in the level of activity *in vivo* of CdR kinase. This decrease probably results from a feedback inhibition of CdR kinase by the relatively large pool of endogenous dCTP present in infected cells. Furthermore, while Ara-C induces an increase in the level of activity of CdR kinase in noninfected cells, it does not do so in infected cells.

It is interesting to note that whereas there is no significant increase in activity of CdR kinase in infected cells incubated with 10 μg/ml of Ara-C, there is a relatively large increase in the activity of this enzyme in extracts of noninfected cells incubated with only 0.2 μg/ml of Ara-C. Since the amount of Ara-C phosphorylated by the cells is directly proportional to the concentration of the drug in the medium (Table 2), and since the infected cells phosphorylate approximately four times less Ara-C than the noninfected cells, it is clear that even when phosphorylated Ara-C is present in the infected cells in amounts which are sufficient to induce an increase of activity of CdR kinase in noninfected cells, this increase does not occur in the infected cells. Thus, not only is the activity of the CdR kinase *in vivo* decreased by infection of RK cells with pseudorabies virus but the mechanism by which an increase in the activity of this enzyme is induced by Ara-C in noninfected cells does not operate in the infected cells.

The drugs, Ara-C and IUdR, have been found to be effective in the control of herpes keratitis. We have shown that, under certain conditions, IUdR is incorporated to a greater degree into the DNA of infected than of noninfected cells, probably as a result of the increase, after infection, in the level of activity of TdR kinase (11, 12). On the other hand, as the results in this paper demonstrate, Ara-C is less effective in inhibiting DNA synthesis in infected than in noninfected cells, probably because of the relatively low level of activity of CdR kinase in these cells as compared to the noninfected cells. Concentrations of the drug which are effective in suppressing viral growth will therefore be toxic to the cells and the drug cannot be considered a specific antiviral agent.

Since the DNA of the viruses of the herpes group does not contain unusual bases, it has been difficult to devise antiviral compounds effective in controlling the diseases caused by these viruses. The difficulty stems from the fact that analogs of the derivatives of DNA will, in principle, be as effective in interfering with the synthesis of cellular DNA as with that of viral DNA. However, infection of cells with viruses of the herpes group induces changes in the level of activity of a variety of enzymes involved in the synthesis of DNA (23). Quantitative differences in the level of activity of these enzymes between infected and noninfected cells may be exploited to provide a useful approach to the chemotherapy of these viruses, since a differential use of pathways for the elaboration of a given component essential to DNA synthesis may result. Thus, the results in this paper show, for example, that whereas CDP reductase activity is increased in infected cells, CdR kinase activity is decreased in these cells. Conditions which would affect DNA synthesis more radically in infected cells than in noninfected cells could thus be created, in principle, by supplying the cells with CdR and with an analog of CR which can be reduced and incorporated into DNA.

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