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Inhibition of DNA Polymerase by β-D-Arabinosylcytosine and Reversal of Inhibition by Deoxycytidine-5′-triphosphate* (32708)

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The antitumor activity of β-D-arabinosylcytosine (ara-c)[2] has been well established (1). One site of action implicated has been inhibition of cytidine diphosphate reductase (2,3). Possible inhibitions by ara-c of the mammalian adenylate and guanylate reductases were also explored (4,5). The cytidine diphosphate and adenylate reductases were found to be inhibited by ara-c (3–5). The inhibitory effects on these 2 reductases, however, were minimal (3,4). In the case of the adenylate reductase, paradoxical drug effects were obtained with enzymes from the Ehrlich and Ca755 ascites tumors (4,5), i.e., inhibitions were reversed with increasing concentrations of drug. Since the ribonucleotide reductases have been shown to be regulatory enzymes for DNA synthesis (5,6,7), presumably, even minimal effects by ara-c on the reductases might eventually lead to profound inhibitory effects on DNA synthesis. Our previous study of the effects of ara-c on the incorporation of glycine-2-^{14}C, adenine-8-^{14}C, and orotic acid-6-^{14}C into the nucleic acids of the Ehrlich ascites tumor had implicated another site of inhibition, namely, the DNA polymerase of the Ehrlich tumor (3). In the study presented here, we have found that ara-c produced inhibitory effects on the utilization of thymidine-^{3}H for DNA synthesis by the Ehrlich ascites tumor. The DNA polymerase was found to be inhibited by ara-c, and this inhibition could be reversed by addition of deoxycytidine-5′-triphosphate to the medium.

Materials and Methods. Random bred female Swiss mice (Simonsen Laboratories, Gilroy, California) were each implanted with 7×10^5 Ehrlich ascites cells and used 5–6 days later for *in vivo* and *in vitro* metabolic studies.

Studies in vivo *and* in vitro. Mice were used for *in vivo* metabolic studies 6 days after implantation of tumor cells. Each mouse received either saline, ara-c, deoxycytidine, or ara-c plus deoxycytidine. One hour later each mouse received thymidine-^{3}H, 3 μmoles, 8.8 $\times$ 10^6 cpm/μmole (Schwarz BioResearch, Inc.) and metabolic utilization was allowed to occur for 1 hour. The fractionation and preparation of the Ehrlich ascites cell DNA has been described in detail elsewhere (3). For *in vitro* studies with Ehrlich ascites cell suspension, Swiss mice with 5-day implants were used as a source of cells. The incubation mixtures in 32 ml of Kreb's original Ringer phosphate medium, pH 7.4 (no $CaCl_2$), contained the following: glucose, 5.5×10^{-3} *M*; thymidine-^{3}H, 5×10^{-4} *M*, 8.8×10^6 cpm/μmole; and Ehrlich cells, 25 mg wet weight per ml of medium. Ara-c, 10^{-3} *M*, dissolved

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[2] The abbreviations used are: β-D-arabinosylcytosine, ara-c-DNA, deoxyribonucleic acid; dCTP, deoxycytidine-5′-triphosphate; TTP, thymidine-5′-triphosphate; TMP, thymidine-5′-monophosphate; ATP, adenosine-5′-triphosphate; dGTP, deoxyguanosine-5′-triphosphate; TCA, trichloroacetic acid; Tris buffer, tris(hydroxymethyl)aminomethane buffer.

in medium was preincubated for 15 min at 37°C before addition of the thymidine-^{3}H. Incubations were carried out in 50 ml Ehrlenmeyer flasks in a metabolic shaker in air at 37°C. At the designated times, 4.0 ml aliquots were removed and added to chilled centrifuge tubes containing 0.44 ml of ice-cold 50% TCA. Each tube was allowed to sit in an ice bath for 2 min, then centrifuged for 2 min at 1470*g*. The acid-wash was discarded and the cells were resuspended in 4.0 ml (80 volumes) of ice-cold 5% TCA and spun down after 2 min. This wash was repeated once more and acid was removed with 2 washes of 4.0 ml each time of ice-cold 95% ethanol. The tissue residue was dissolved in 2.0 ml of hyamine hydroxide at room temperature in the dark overnight. Aliquots of 0.5 ml were counted in a scintillation solution at 6% efficiency.

"DNA polymerase." The preparation of enzyme extracts of the Ehrlich ascites tumor has been described (3). The incubation tubes (in duplicate) each contained TTP-2-^{14}C, 10 mμmoles, 3.3 $\times$ 10^6 cpm/μmole (New England Nuclear) or TMP-2-^{14}C, 10 mμmoles, 14.4 $\times$ 10^6 cpm/μmole (New England Nuclear); Tris buffer, pH 7.8, 44 μmoles; $MgCl_2$, 6 μmoles; creatine phosphate, 5 μmoles; creatine phosphokinase, 75 μg; dGTP, dATP, and dCTP, 20 mμmoles each; ATP, 1 μmole; denatured DNA, 100 μg; and Ehrlich enzyme extract, 1.72–2.00 mg protein, in a final volume of 0.70–0.80 ml. The analysis for incorporation of TMP-2-^{14}C (or TTP-2-^{14}C) into an acid-insoluble product, presumably DNA, has been described (8). Protein was determined by the method of Lowry *et al.* (9).

Results. Studies in vivo *and* in vitro. The *in vitro* incorporation of thymidine-^{3}H into an acid-insoluble fraction of Ehrlich ascites cells, presumably DNA, was depressed 70–80% by ara-c at 10^{-3} *M* (Table I). Ara-c at 60 mg/kg inhibited by 99% the *in vivo* incorporation of thymidine-^{3}H into the DNA of the Ehrlich tumor (Table II). Deoxycytidine, 60 mg/kg, equimolar with ara-c at 60 mg/kg, only partially reversed the inhibition produced by ara-c *in vivo*.

"DNA polymerase": Table III shows various parameters affecting the Ehrlich tumor "DNA polymerase." The incorporation of TTP-2-^{14}C into an acid-insoluble product, presumably DNA, was absolutely dependent on DNA primer. Deletion of dATP, dGTP, and dCTP decreased enzyme activity about 50%. The addition of dCTP in 6-fold excess did not significantly increase activity. The incorporation of TTP-2-^{14}C was linear for at least 30 min. The ara-c, 1.25 $\times$ 10^{-4} *M*, inhibited the "polymerase" and this inhibition was reversed by dCTP at 1.75 $\times$ 10^{-4} *M*.

TABLE I. *In Vitro* Inhibition of Thymidine-^{3}H Incorporation into Ehrlich Ascites Cell Suspensions by ara-c.[a]

Incubation time (min)	cpm/gm cells		Inhibition (%)
	Control	+ 10^{-3} *M* ara-c	
0	0	0	—
10	5040	720	86
20	6120	1680	72
30	6720	960	86
40	8760	2040	77

[a] The incubation medium in Kreb's original Ringer phosphate buffer, pH 7.4, (no $CaCl_2$), 32 ml, in 50-ml Ehrlenmeyer flasks contained the following: glucose, 5.5 $\times$ 10^{-3} *M*; thymidine-^{3}H, 5 $\times$ 10^{-4} *M*, 8.8 $\times$ 10^6 cpm/μmole and Ehrlich ascites cells, 25 mg wet wt./ml of medium. The ara-c, dissolved in medium, was preincubated at 37°C for 10 min with cells before the addition of the thymidine-^{3}H.

TABLE II. *In Vivo* Inhibition of Thymidine-^{3}H Incorporation into DNA Thymine by ara-c.[a]

Drug	DNA (cpm/ml cells)	DNA thymine (cpm/μmole)
Saline	50,600	8,540
ara-c, 60 mg/kg	567	79
deoxycytidine, 60 mg/kg	53,200	10,316
deoxycytidine, 60 mg/kg + ara-c, 60 mg/kg	5,860	1,189

[a] Female Swiss mice, 31–32 gm, bearing 6-day implants of the Ehrlich ascites tumor were each given saline ara-c, deoxycytidine, or ara-c plus deoxycytidine. One hour later each mouse received thymidine-^{3}H, 3 μmoles, 8.8 $\times$ 10^6 cpm/μmole and metabolic utilization was allowed to occur for 1 hour. Each value is the average of separate determinations on 3 mice.

TABLE III. "DNA Polymerase" Activity in Enzyme Extracts of the Ehrlich Ascites Tumor.[a]

System	Incubation time (min)	mμmoles TTP-2-^{14}C incorporated
Complete	0	0
	10	0.36
	20	0.62
	30	0.87
	15	0.46
Minus ½ enzyme	15	0.24
Minus dGTP, dATP, dCTP, 20 mμmoles each	15	0.19
Minus DNA primer	15	0.00
Plus ara-c, 100 mμmoles	15	0.27
Plus dCTP, 120 mμmoles	15	0.54
Plus ara-c, 100 mμmoles, and dCTP, 120 mμmoles	15	0.55

[a] The complete system contained TTP-2-^{14}C, 10 mμmoles, 3.3×10^6 cpm/μmole; Tris buffer, pH 7.8, 44 μmoles; $MgCl_2$, 6 μmoles; ATP, 1 μmole; creatine phosphate, 5 μmoles; creatine phosphokinase, 75 μg; dGTP, dATP, and dCTP, 20 mμmoles each; denatured DNA, 100 μg; and Ehrlich enzyme extract, 1.72 mg protein, in a final volume of 0.80 ml. Ara-c, 100 mμmoles; dCTP, 120 mμmoles; or H_2O was preincubated with enzyme and all reagents except TTP-2-^{14}C, dGTP, dATP, and dCTP, 20 mμmoles each, for 10 min at 37°C, then these were added and incubations continued.

The results of further studies on inhibition of the Ehrlich tumor "DNA polymerase" are given by data presented in Table IV. The ara-c, 1.43×10^{-4} *M* and 2.86×10^{-4} *M*, produced a 70–75% inhibition of incorporation of TTP-2-^{14}C or TMP-2-^{14}C into the acid-insoluble product. This inhibition of the Ehrlich "DNA polymerase" by ara-c was reversed by dCTP even when the molar ratio of ara-c to dCTP was 2.86 to 1 (Table V).

Discussion. The ara-c (or metabolites) was found to inhibit the *in vivo* and *in vitro* utilization of thymidine for DNA synthesis in the Ehrlich ascites tumor. These data implied that ara-c might be inhibiting the DNA polymerase of this mammalian tumor. Previously, we had conjectured on this possible site of action based on studies of incorporations of various radioactive precursors into the DNA of the Ehrlich tumor (3). Our present finding that the "DNA polymerase" of the Ehrlich tumor was inhibited by ara-c (or metabolites) was not unexpected. Since ara-c could be considered an analog of deoxycytidine, reversal of ara-c inhibition of the "DNA polymerase" by dCTP was also not unexpected. The mechanaism of reversal by dCTP is not known but several possibilities can be listed. (a) The dCTP (or metabolites) might have inhibited the conversion of ara-c to ara-CTP. (b) The dCTP might have com-

TABLE IV. Inhibition of Ehrlich Ascites Tumor "DNA Polymerase" by ara-c.[a]

ara-c (*M*)	Incubation time (min)	Precursor	Incorporated (mμmoles)	Inhibition (%)
None	15	TTP-2-^{14}C	0.78	—
1.43×10^{-4}	15	TTP-2-^{14}C	0.25	68
2.86×10^{-4}	15	TTP-2-^{14}C	0.20	74
None	15	TMP-2-^{14}C	0.37	—
1.43×10^{-4}	15	TMP-2-^{14}C	0.12	67
None	30	TMP-2-^{14}C	0.69	—
1.43×10^{-4}	30	TMP-2-^{14}C	0.21	69
None	45	TMP-2-^{14}C	0.86	—
1.43×10^{-4}	45	TMP-2-^{14}C	0.20	77

[a] The incubation tubes each contained TTP-2-^{14}C, 10 mμmoles, 3.3×10^6 cpm/μmole, or TMP-2-^{14}C, 10 mμmoles, 14.4×10^6 cpm/μmole; Tris buffer, pH 7.8, 44 μmoles; $MgCl_2$, 6 μmoles; ATP, 1 μmole; creatine phosphate, 5 μmoles; creatine phosphokinase, 75 μg; dGTP, dATP, and dCTP, 20 mμmoles each; denatured DNA, 100 μg; and Ehrlich enzyme extract, 2.00 mg protein, in a final volume of 0.70 ml. Ara-c or H_2O was preincubated with enzyme and all reagents except TTP-2-^{14}C or TMP-2-^{14}C, dGTP, dATP, and dCTP, 20 mμmoles each, for 10 min at 37°C, then these were added and incubations continued.

TABLE V. Reversal of ara-c Inhibition of "DNA Polymerase" by dCTP.[a]

ara-c (M)	TTP-2-^{14}C incorporated (mμmoles)		Molar ratio
	Control	$+ 1.75 \times 10^{-4} M$ dCTP	ara-c to dCTP
None	0.41	0.52	0.00:1
6.25×10^{-5}	0.22 (46)	0.46 (11)	0.36:1
1.25×10^{-4}	0.19 (54)	0.48 (8)	0.72:1
2.50×10^{-4}	0.17 (58)	0.50 (4)	1.43:1
5.00×10^{-4}	0.16 (61)	0.47 (10)	2.86:1

[a] The incubation tubes each contained TTP-2-^{14}C, 10 mμmoles; 3.31×10^6 cpm/μmole; Tris buffer, pH 7.8, 44 μmoles; $MgCl_2$, 6 μmoles; ATP, 1 μmole; creatine phosphate, 5 μmoles; creatine phosphokinase, 75 μg; dGTP, dATP, dCTP, each 20 mμmoles; denatured DNA, 100 μg, and Ehrlich enzyme extract, 1.72 mg protein; in a final volume of 0.80 ml. Ara-c, various molarities; dCTP, 120 mμmoles, or H_2O was preincubated with enzyme and all reagents except TTP-2-^{14}C, 10 mμmoles; dGTP, dATP, and dCTP, 20 mμmoles each at 37°C for 10 min, then these were added and incubations continued for 15 min. Numbers in parentheses refer to percentage inhibition.

peted with ara-c (or metabolite) for the same binding site on the DNA polymerase. (c) The binding of dCTP to the polymerase might have changed the conformation of the enzyme so that ara-c (or a metabolite) could not bind to an allosteric inhibitor site. These possibilities are subjects for future investigations.

The reversal of ara-c activity *in vivo* by deoxycytidine was first shown by Evans and Mengel (10). Our results may explain, in part, the results of Evans and Mengel (10).

Summary. β-D-Arabinosylcytosine was found to inhibit the utilization of thymidine for DNA synthesis in the Ehrlich ascites tumor. The DNA polymerase of the Ehrlich tumor was inhibited by β-D-arabinosylcytosine (or metabolite) and this inhibition could be reversed by deoxycytidine-5'-triphosphate.

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Formation of Inosine-5'-Monophosphate by a Kinase in Cell-Free Extracts of Ehrlich Ascites Cells *in Vitro** (32709)

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The effects of purine nucleosides upon the metabolism of normal and neoplastic tissues have led to increased investigations into the means by which these compounds exert their actions. Recent studies have shown that the active forms of the cytotoxic analogs of purine bases and nucleosides are the nucleotides, which are formed intracellularly by the action of pyrophosphorylases and kinases, respec-

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