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2′-Deoxycytidine Hydrochloride Protection of Mice Against the Lethal Toxicity of Cytosine Arabinoside

Absence of Protection of Bone Marrow Lymphocytes*

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Summary. *Normal mice and mice with advanced leukemia were injected IP with cytosine arabinoside (araC) on a lethal treatment schedule. The simultaneous oral administration of deoxycytidine (CdR) in doses 2–3 times as high as those of araC prevented drug-related death of the animals. Although CdR protected myelo- and erythropoiesis in the bone marrow of normal mice treated with araC, it did not influence the suppression of bone marrow lymphopoiesis. However, in mice treated with araC + CdR, the peripheral blood levels of both lymphocytes and granulocytes were significantly higher than those in animals given araC only. AraC lowered the level of serum hemagglutinins in mice immunized with sheep red blood cells (SRBC), and prolonged skin allograft survival. CdR appeared to normalize only the cellular immune response. In mice with advanced L1210 lymphoblastic leukemia treated with araC + CdR, the effect of the metabolite was selective, i.e., CdR administration led to a complete abrogation of drug-induced lethality without interfering with the highly efficient therapeutic effect of araC. No antitumor effect of araC was shown in araC + CdR-treated mice with advanced granulocytic Graffi leukemia and erythroblastic Rauscher leukemia. We suppose that the combined effect of araC and CdR, araC being administered on a lethal treatment schedule, results in selective destruction of precursor cells with the emphasis on B lymphocytes.*

Introduction

The search for new drugs or combination of drugs selectively inhibiting the proliferation of various cell types is of great theoretical and practical value. One approach in this area is to improve therapeutic efficacy by combining a metabolite with its corresponding synthetic antimetabolite [1, 2, 4–6, 14, 15, 22, 26, 28–31].

Toxicity of araC can be prevented, reduced, or reversed by CdR under certain conditions. The maximal level of protection is achieved by injection of CdR simultaneously with araC in doses 2–4 times higher; however, no selective effect is observed in this case. Venditty and Goldin reported [31] that in the case of early L1210 lymphoblastic leukemia the CdR + araC combination did not show any therapeutic advantage over araC alone given in optimal doses. Nevertheless, they were able to achieve complete prevention of lethal toxicity together with the marked therapeutic effect.

Here we report the results of experiments in normal, immunized, and leukemia-bearing mice. The data obtained lead us to suggest that the combination of lethal doses of araC with CdR protection results in the selective destruction of B lymphocytes and/or B cell precursors.

Materials and Methods

Animals. Two- to three-month-old C57 Bl/6j (B6), BALB/c (C), DBA/2 (D2), (♂ DBA/2 × ♀ C57 Bl/6j) F1♂ (B6D2F1) mice obtained from the Stolbovaya breeding farm of the USSR Academy of Medical Sciences and outbred white mice obtained from the Krjukovo breeding farm of the Academy were used.

Drugs. CdR (Reanal, Hungary) and araC (Upjohn, USA) were dissolved in saline and injected on the same day. All inoculations were performed with 0.2 ml per mouse, including drugs and cells. CdR was administered by oral intubation, while araC was co-administered IP. In most experiments the drugs were given three to four times a day at 2-h intervals.

Study of Toxicity. The toxic effect was evaluated by such parameters as the frequency of mortality and of diarrhea, changes in body weight and in weight and cellularity of the spleen, and total and differential peripheral blood and bone marrow nucleated cell counts. The total number of nucleated cells was estimated per cubic millime-

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ter of peripheral blood and per femur. The smears of blood and bone marrow were stained by the method of Romanovsky and the percentage of different cell types was calculated after analysis of 200 cells in the blood smears and 500 cells in the bone marrow smears.

Study of the Humoral and Cellular Immune Response. The titers ($\log_2$) of serum hemagglutinins (SHA) in B6 mice immunized IP with 2.5×10^8 SRBC were estimated in a Takatchi microtitrator (Labor MSM, Hungary). The skin from the back of donor C females was grafted onto the back of B6 recipient females by the method of Medawar. Starting from day 7, the mice with the skin graft were examined for rejection, and the day of complete rejection was noted ($\geqslant 90\%$ necrosis).

Study of Antileukemic Effect. Lymphoblast L1210 leukemia, myeloblast Graffi (MG) leukemia [9] and erythroblast Rauscher (ER) leukemia were maintained in D2 males, B6, and C females, respectively, by IP (L1210, ER leukemias) and IV passaging (MG leukemia). In our experiments 10^5 L1210 ascites tumor cells were inoculated SC. Spleen nucleated cells (5×10^3) from mice bearing MG leukemia were inoculated IV, and 2×10^8 spleen nucleated cells from mice bearing ER leukemia were inoculated IP. The antitumor effect was evaluated by the mean survival time (MST) and the percentage increase in lifespan was calculated (%ILS). In some experiments with ER leukemia the splenomegaly level was noted. All dead animals were autopsied to determine macroscopic symptoms of leukemia. In a number of cases the spleen, liver, and subcutaneous tumor (in the case of L1210 leukemia) were weighed. The data obtained showed whether the mice concerned died of leukemia or of drug toxicity.

Statistics. For each group of animals the mean and standard error (SE) were calculated. The means were compared by Student's t-test. A significance level of $P \leqslant 0.05$ was chosen.

Results

A special study of the effect of oral or IP CdR administration both in a single dose schedule and given daily for 8 days in single or fractional doses showed that it was safe to hybrid and inbred mice. No toxic, immunosuppressive, antileukemic, or stimulating effects of the metabolite were observed. This finding is consistent with the data reported by other investigators [3, 7, 8, 10–13, 27]. In a number of cases our data on the effect of CdR alone are therefore omitted.

Oral co-administration of CdR in B6 or D2 mice with IP lethal injections of araC LD_{70-100} prevented the drug-related death of mice by diminishing various toxic effects (Fig. 1). Certain peculiarities of the protective effect of CdR with respect to hematopoiesis should be pointed out. In all groups of mice receiving araC, a striking decrease in the levels of all nucleated cell types in peripheral blood was observed (Fig. 1). However, even the lowest levels of circulating leukocytes, both lymphocytes and granulocytes, occurring 3–4 days from the beginning of treatment, were significantly higher in CdR-protected mice than in those treated with araC alone. But in the bone marrow on the next day

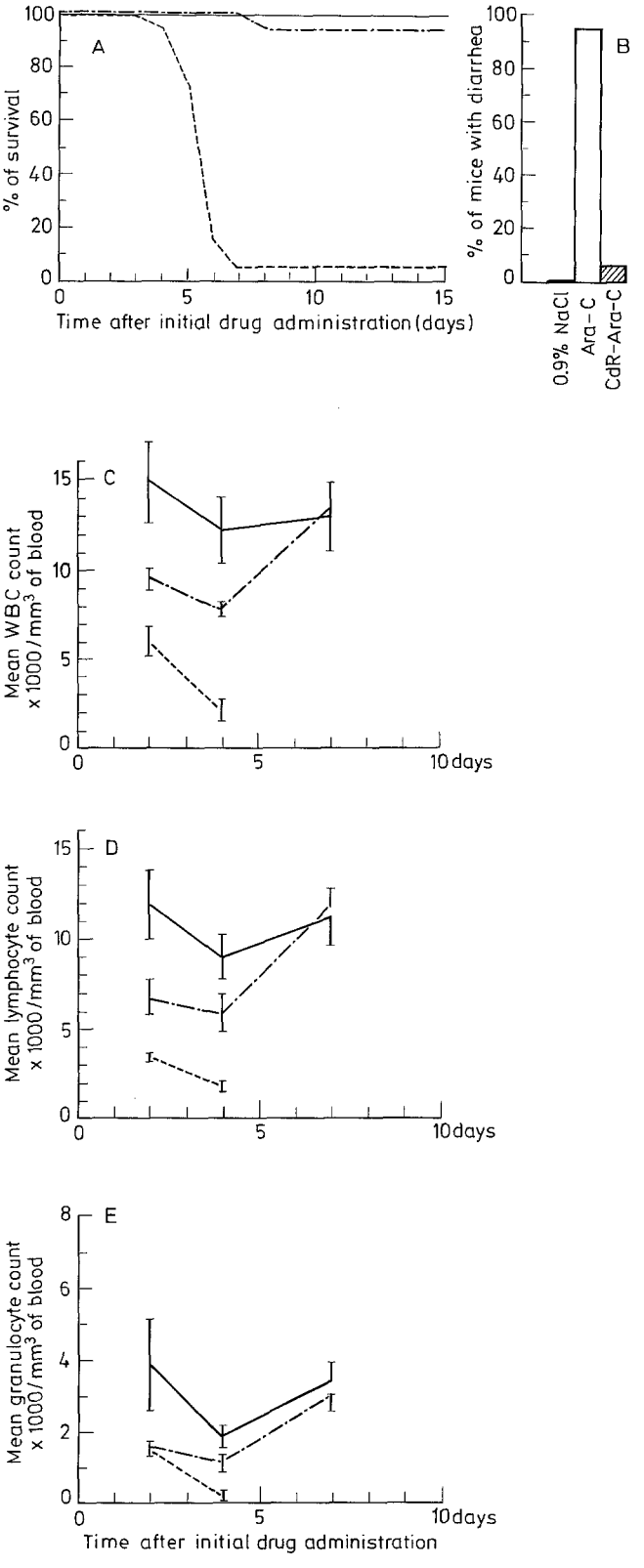

Fig. 1. The influence of CdR on general and hematological toxicity of araC. B6 females were injected simultaneously with CdR PO in a dose of 40 mg/kg and araC IP in a dose of 20 mg/kg three times a day from day 0 to day 4. Experimental groups contained 5–20 mice. ——, physiological saline; - - - -, araC; —·—·— CdR + araC; – – – –, CdR

Table 1. The influence of CdR on bone marrow toxicity of araC

Group no.[b]	Cells	Average no. of cells (× 10^6) in the femur (mean ± SE)		
		Saline (a)	araC (b)	CdR + araC[a] (c)
1	Lymphocytes	6.13 ± 0.82	3.17 ± 0.72	3.62 ± 0.29
2	Reticular cells	1.19 ± 0.09	0.42 ± 0.12	1.00 ± 0.17
	Hemocytoblasts	0.33 ± 0.08	0.08 ± 0.02	0.19 ± 0.06
	Mitotic granulocytes[c]	0.95 ± 0.14	0.47 ± 0.09	1.15 ± 0.18
	Mitotic cells	0.23 ± 0.07	0.05 ± 0.02	0.22 ± 0.06
3	Postmitotic granulocytes[d]	8.68 ± 1.00	0.75 ± 0.15	4.59 ± 0.42
	Erythron[e]	8.31 ± 1.38	1.41 ± 0.43	4.30 ± 0.83
4	Total nucleated cell count	26.10 ± 2.19	6.60 ± 1.33	15.60 ± 1.22

[a] CdR was administered to B6 females PO in a dose of 40 mg/kg three times a day for 4 days, araC was injected IP in a dose of 20 mg/kg simultaneously. Bone marrow was obtained the day after completion of the course

[b] Cell types from group 1 were not protected ($P_{a-c} < 0.05$; $P_{b-c} > 0.05$); cell types from group 2 were completely protected ($P_{a-c} > 0.05$; $P_{b-c} < 0.05$–0.01); those from groups 3 and 4 were partially protected ($P_{a-c} < 0.05$–0.01; $P_{b-c} < 0.05$–0.001). Each group contained not less than five mice. The table includes data on cell types that significantly reduced in number in araC treated mice ($P < 0.05$–0.001)

[c] Myeloblasts + promyelocytes + myelocytes

[d] Metamyelocytes + bands + segmented granulocytes

[e] Nucleated forms of red cells

Table 2. The influence of CdR on inhibition of humoral antibody response and toxicity induced by lethal doses of araC[a]

Groups	Daily doses of drugs (mg/kg)		$1/n$[b]	The titer ($\log_2$) SHA ($\bar{M}$ ± SE)	
	CdR	araC		Day 5	Day 8
1	0	0	0/15	5.6 ± 0.6	5.5 ± 0.5
2	0	60	15/15	1.8 ± 0.3	[c]
3	120	60	0/10	1.6 ± 0.3	2.6 ± 0.9
4	180	60	0/10	1.9 ± 0.3	1.8 ± 0.8
5	120	0	0/10	3.4 ± 0.6	4.2 ± 1.4
6	180	0	0/10	4.5 ± 0.6	5.3 ± 0.6

On day 5, Group 1 vs Groups 2, 3, 4: $P < 0.01$; Group 2 vs Groups 3, 4: not significant, and Group 5: $P < 0.05$; on day 8, Group 1 vs Group 3: $P < 0.05$, and Group 4: $P < 0.01$

[a] B6 females were immunized with 2.5 × 10^8 SRBC IP on day 0; the drugs were given three times daily on days 1–4

[b] l, number of mice that died; n, total number of mice in the group

[c] All mice receiving araC alone died within 5–7 days

Table 3. The influence of CdR on prolongation of skin allograft survival and toxicity induced by lethal doses of araC[a]

Daily doses of drugs (mg/kg)		$1/n$[b]	The time of complete rejection (days) (mean ± SE)
CdR	araC		
0	0	0/8	10.0 ± 0.38 (8–11)[c]
120	0	0/7	9.7 ± 0.56 (8–11)[c]
0	60	4/6	16[d]
120	60	0/8	10.5 ± 0.19 (10–11)[c]

[a] On day 0 the skin from C mice was grafted in B6 females; the drugs were administered starting from next day 3 times a day daily for 5 days

[b] See footnotes to Table 2

[c] Figures in parentheses indicate the range

[d] In two surviving mice the skin allograft was rejected on day 16

after the end of the treatment the lymphocytes in contrast to other cells, were equally decreased in numbers in mice receiving araC alone and in those receiving araC in combination with CdR (Table 1). Despite the fact that araC alone reduced the number of bone marrow lymphocytes only twofold, CdR did not influence this relatively weak effect. This evidence leads us to suggest that it is possible to destroy certain functions of the lymphoid system selectively by means of araC administered in lethal doses under CdR protection. The results of the first experiments carried out to test this possibility are reported below. At this point it is worth while to stress that CdR significantly protected the proliferating granulocytic cells, the direct targets for the cytotoxic effect of araC. However, the degree of protection of these cells was more pronounced than that of their mature off-

Table 4. The influence of CdR on antitumor effect and toxicity of araC in the treatment of advanced lymphoblast L1210 leukemia[a]

Expt. no.	Daily doses of drugs (mg/kg)		Regimen of treatment	MST ± SE (days)	ILS (%)	s/n[b]	Changes in body weight (g) after courses		Suggested cause of death[c]
	CdR	araC					1	2	
1	0	0	3 times	9.6 ± 0.3		0/18	2.8	–	L
	0	12	a day on days	22.8 ± 0.6	138	0/9	− 1.2	− 1.0	L
	360	120	5–7, 11–13, 18–20	24.6 ± 0.7	156	0/17	− 0.9	− 1.2	L
2	0	0	4 times	15.0 ± 0.4		0/10	0.5	1.2	L
	480	0	a day on days	15.3 ± 0.5	2	0/10	0.6	0.7	L
	0	160	5–7, 12–14, 19, 20	10.0 ± 0.1	− 50	0/32	− 3.0	–	T
	480	160		38.4 ± 1.3	156[d]	8/35	− 1.5	− 1.0	L
3	0	0	4 times	10.4 ± 0.2		0/11	1.4	–	L
	480	0	a day on days	10.6 ± 0.2	1	0/11	− 0.2	–	L
	0	160	8, 9, 13–15	15.6 ± 0.8	50	0/11	− 5.7	− 9.2	T
	480	160		20.7 ± 0.3	99	0/9	−10.4	−12.8	L?T?
4	0	0	4 times	14.6 ± 0.2		0/30	1.6	–	L
	48	0	a day on days	14.2 ± 0.4	− 3	0/8	0.9	–	L
	480	0	10–18[e]	14.1 ± 0.2	− 3	0/20	1.6	–	L
	0	16		26.7 ± 0.3	83	0/10	− 0.9	− 1.7	L
	0	160		18.4 ± 0.1	26	0/20	− 0.4	− 5.2	T
	48	16		23.1 ± 0.3	58	0/9	− 0.4	− 1.0	L
	480	160[f]		20.3 ± 0.3	39	0/7	− 0.7	− 6.1	T
	480	160[f]		27.7 ± 0.4	90	0/12	− 0.6	− 4.3	L

[a] 10^5 ascites L1210 cells were inoculated SC in B6D2F1 males on day 0 in Expts. 1, 2, and 4 and in D2 females in Expt. 3.
[b] *s*, number of mice that survived for over 60 days; *n*, total number of mice in the group
[c] L, leukemia-related death; T, drug-related death
[d] Mice that survived for over 60 days are not included
[e] In Expt. 4, mice from all groups except Group 1 received CdR in daily doses of 48 mg/kg and araC in daily doses of 16 mg/kg, then the mice from Groups 2, 4, and 6 continued to receive the drugs in the same doses up to day 17, and the mice from Groups 3, 5, 7, and 8 received doses escalated 10-fold from day 13 to day 18
[f] 7 of 19 mice treated with a high-dose CdR + araC combination developed lethal toxicosis, so the mice are divided into two subgroups

spring. Possibly this is connected with the accelerated rate of proliferation in the case of unstable hematopoiesis and/or with the degree of proliferating granulocytic cell inhibition at earlier times (days 2 and 3 after the beginning of treatment).

CdR inoculation protected B6 mice immunized with SRBC (Table 2) and the recipients of skin allografts (Table 3) against the lethal toxicity of araC. Mice treated with araC alone developed immunosuppression as a result of the severe toxicity of the drug. Suppression of the immune response to SRBC in mice treated with the CdR + araC combination was selective, i.e., it took place in the absence of toxic effects. At the same time, CdR inhibited delayed allogeneic skin graft rejection induced by araC.

CdR inoculation also reduced the toxicity of araC in mice with advanced leukemia. The survival of L1210 leukemia-bearing mice in the group treated with CdR + araC was significantly prolonged as compared with that of other groups (%ILS $\geqslant$ 100). The %ILS of mice bearing MG and ER leukemia appeared to be low (Tables 4, 5). AraC administration led to a significant decrease in the spleen weight of mice with ER leukemia; this effect was reversed by CdR (Fig. 2). According to the data obtained in our laboratory [9] and to unpublished observations, a nontoxic dosage of araC exerts a meager therapeutic effect on MG and ER leukemia.

Discussion

The protective effect of CdR is accounted for by its competing with araC for certain enzymes of NA biosynthesis [23] and for carrier of the transport system responsible for the cell uptake of those drugs [24]. The possibility of diminishing the toxicity of araC safely by means of CdR in different systems in vivo and in vitro has already been demonstrated (see Introduction). Analysis of the reported data demonstrates two major peculiarities of CdR protection against the lethal toxicity of araC. Firstly, CdR inoculation results in defense of GIT cells, granulocytopoiesis, and erythropoiesis in the bone marrow, and this defense is sufficient for a good life level. However, less markedly affected bone marrow lymphopoiesis remains unprotected. Secondly, when

Table 5. The influence of CdR on antitumor effect and toxicity induced by lethal doses of araC in the treatment of advanced nonlymphoid types of leukemia[a]

Expt. no.	Daily doses of drugs (mg/kg)		Days of drug administration	MST ± SE (days)	ILS (%)	Suggested cause of death[c]	n^b
	CdR	araC					
1	0	0	10–12, 15, 16	16.2 ± 0.3		L	9
	480	0		16.1 ± 0.4	− 1	L	11
	0	160		14.2 ± 0.2	−12	T[d]	11
	480	160		19.0 ± 2.7	17	L	10
2	0	0	16, 17	26.3 ± 4.2		L	7
	480	0		34.0 ± 5.7[e]	29	L	8
	0	160		21.6 ± 1.0	−18	T?L?	8
	480	160		22.0 ± 0.7	−16	L	8
3	0	0	15, 16, 20,	24.7 ± 2.4		L	7
	480	0	21, 25, 26	24.3 ± 1.9	− 2	L	10
	0	160		25.8 ± 1.6	4	T[f]	10
	480	160		27.0 ± 2.1	9	L	6

[a] In Expt. 1, 5×10^3 spleen cells from mice bearing MG leukemia were inoculated IV on day 0 to B6 males. In Expts. 2 and 3, 2×10^8 spleen cells from mice bearing ER leukemia were inoculated IP on day 0 to C females. AraC was injected IP in a dose of 40 mg/kg four times a day; CdR was co-administered PO in a dose of 120 mg/kg four times a day.

[b] Number of mice in the group

[c] See footnotes to Table 4

[d] 10 of 11 mice developed lethal toxicity and died on days 14 or 15, one mouse did not develop toxicosis and died on day 20.

[e] 1 of 8 mice was killed on the day of the cessation of experiment (day 60), due to marked splenomegaly

[f] The majority of mice developed lethal toxicosis after 25 and 26 days of treatment

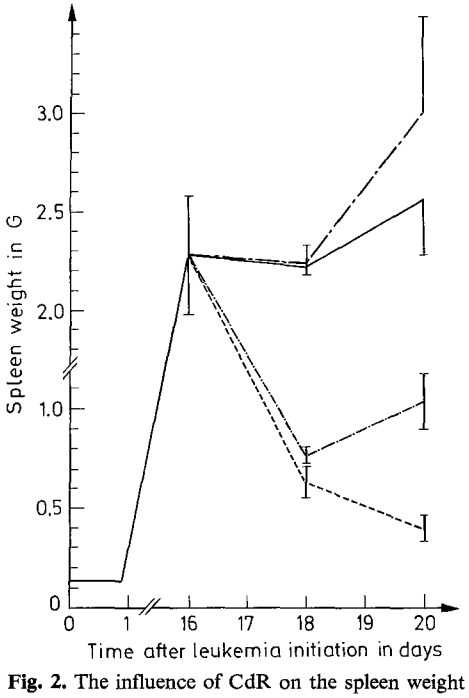

Fig. 2. The influence of CdR on the spleen weight in mice with ER leukemia. Summarized data of two experiments. C mice were inoculated IP with ER leukemia cells on day 0. On days 16 and 17 they were injected simultaneously with CdR PO in a dose of 120 mg/kg and araC IP in a dose of 40 mg/kg four times a day. ——, physiological saline; - - -, araC; — · —, CdR + araC; — — — CdR

CdR is administered, araC can destroy only certain lymphocyte subpopulations. Thus, the level of blood lymphocytes, in contrast to that of bone marrow lymphocytes, was significantly higher in mice protected against toxicity, although it was under the norm within 2–4 days of drug inoculation. 'Protected' mice retained the capactity for skin allotransplant rejection; this indicates that certain lymphocyte subpopulations are protected. At the same time, other lymphocyte subpopulations are destroyed, as shown by the undiminished immunosuppressive effect of araC in SRBC-immunized protected mice.

A pronounced antitumor effect in protected mice bearing advanced L1210 leukemia indicates that the malignant lymphoblasts have their origin in a lymphoid cell subpopulation that is unprotected by CdR.

Further studies are required to show what particular lymphocyte subpopulations are protected or not protected against the cytotoxicity of araC by CdR. We suggest that the latter refer to B cells. Interestingly, inoculation of araC alone in less nontoxic doses than those used in our experiments also results in the selective inhibition of humoral but not cellular immune response [16–20]. Presumably physiological levels of CdR are not sufficient to protect all lymphocyte subpopulations. According to the data recorded in our laboratory, araC admin-

istered according to a nontoxic schedule leads to statistically significant, though weak, ILS of mice bearing advanced MG [9] and ER leukemias. However, when CdR was applied in the treatment of these forms of nonlymphoid leukemias it protected against the lethal toxicity of araC without favoring its strong antitumor effect. The data obtained by the spleen weight test directly indicate that araC had a reduced antitumor effect when applied simultaneously with CdR. Apparently CdR lessens the cytotoxicity of araC to nonlymphoid malignant cells of these leukemias, as observed in normal analogs of these cells.

Thus, CdR protection of the animal against the lethal toxicity of araC is accompanied by selective destruction of a certain lymphocyte subpopulation.

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