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Effects of Tilorone on Hemopoietic Stem Cells and on the Development of Friend Leukemia

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Summary. *Hematological effects of tilorone, an interferon inducer, on the hematopoietic cell system of normal CBA/Ca mice and on the development of Friend virus (FV-P)-induced polycythemia in DBA/2 mice were studied. In normal mice 80 mg/kg IP had a marked depressive effect on pluripotent (CFU-S), granuloid committed (CFU-C), and erythroid committed (CFU-E) stem cells with regeneration between days 5 and 12. In bone marrow smears only lymphopenia was detected. Treatment of mice before FV-P infection caused a slight retardation in the development of the splenomegaly and the transformation of bone marrow cells to Ep independence. Repeated treatment after FV-P infection also reduced the increase in spleen weight and the development of reticulocytosis, but the Ep independence of bone marrow and spleen cells was not influenced. In vitro exposure of normal cells and cells from FV-P-infected animals to the drug showed the same sensitivity of colony growth in normal as well as in Ep-independent CFU-E. The action of the drug on Friend leukemia is at least in part considered a toxic effect on the hematopoietic stem cell system.*

Introduction

Tilorone (fluoren-9-one, 2,7-bis[2-(diethylamino)-ethoxy]-,dihydrochloride) is known as a substance with a wide spectrum of biological activities. It is a potent inducer of interferon [5, 19], inhibits T cells and T-cell functions [15, 16, 34], enhances B-cell responses [6, 20, 21] and has antiviral as well as cytotoxic activity in several test systems [1, 13, 21, 25, 31]. In clinical studies in man, tilorone was tested without clear effects on tumor growth [26, 32], and no depression of the bone marrow function was reported.

In the experimental studies with this drug the Friend virus-induced leukemia was used by several investigators [2, 21, 24, 25] and an inhibition of the splenomegaly was reported, especially when the drug had been given before the virus infection. Such effects have also been reported after administration of other inducers of interferon, such as poly IC [7, 14], or with interferon itself [10, 11, 33]. But there was no correlation between interferon induction and viral protection [8] and the finding that tilorone also inhibits RNA-dependent DNA polymerase [27] is of special interest for its effect on tumors caused by oncorna viruses.

In recent studies we have reported on the chemotherapy of established Friend leukemia, including the toxicity of the drugs on normal hematopoietic stem cells and the Friend virus (polycythemic strain)-specific erythropoietin-independent erythroid committed stem cells (CFU-EI) [28, 29]. These CFU-EI proved to be a very sensitive marker for the leukemic cell population. We now included tilorone in these experiments and also studied its effects on normal stem cells in the mouse. This was also done in view of recent observations that interferon had a suppressive effect on erythroid cell proliferation [23].

Materials and Methods

1. Mice

Female CBA/Ca (Zentrale Tierversuchsanlage, University of Ulm) and DBA/2 mice (Gl. Bomholtgard, Ry, Denmark) 19–22 g in weight were used. Ten animals per cage were kept in artificial light for 12 h daily. They were fed commercial pellets and water ad libitum.

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2. Virus

The Friend virus, polycythemic strain (FV-P) was kindly supplied by Prof. Schäfer (Tübingen/Germany). It was serially passaged in NMRI and during the last 2 years in DBA/2 mice. The mice were infected IP with 0.25 ml cell-free 10% homogenate of leukemic spleens (2–3 g in weight) in saline.

3. Experimental Procedures

Four mice were used per point. They were killed by cervical dislocation, the spleen weight was determined, and a single cell suspension of the spleens and one femur of each mouse were prepared in α medium (Flow Lab.) containing 2% fetal calf serum (Seromed). Blood for hematological studies was taken from the retro-orbital sinus and studied by standard procedures. Data are given as means ± SD. Bone marrow differentials were analysed, 500 cells per animal, after staining of the smears with May-Grünwald-Giemsa solution. E_1–E_5 were erythroblasts at the different stages of maturation, M_{1-4} were myeloblasts, promyelocytes, and myelocytes – the proliferating granulocytic precursors – and M_{5-8} were the more mature, nonproliferating granulocytic cells. Further lymphocytes and other cells (megakaryocytes, reticulum cells etc.) were classified.

4. CFU-S Experiments

The procedure described by Till and McCulloch [30] was used to assay marrow and spleen colony-forming units (CFU-S). The pooled marrow or spleen cells were injected intravenously ($2-20 \times 10^4$ marrow cells and $1-2 \times 10^6$ spleen cells in 0.25 ml) into irradiated recipients (10 animals per group). Radiation consisted of 800 rads exposure, 280 KV, 12 mA, 1.6 mm Cu and 1 mm Al filter, focal distance 50 cm, rate 30 R/min. Nine days after injection of cells the recipient animals were killed; their spleens were removed and placed in Bouin's solution, in preparation for counting macroscopic colonies after 24 h in the fixative.

5. CFU-C Experiments

Cells from bone marrow or spleen were incubated in 3.5-cm plastic dishes containing 1 ml α medium with 20% horse serum (Seromed) and 3% agar. Colony growth was stimulated by the use of heat-inactivated serum obtained from NMRI mice 3 h after IV injection of 50 µg endotoxin (*Salmonella abortus equi*, Difco). Optimal stimulation was obtained by adding 12.5 µl endotoxin-activated serum. The cell number per plate was $1-2 \times 10^5$ for bone marrow cells and $1-2 \times 10^6$ for spleen cells. The cultures were done in triplicate and incubated in 5% CO_2 at 37° C in a humidified atmosphere. After 7 days of incubation the colonies (> 50 cells being a colony) were counted with 40-fold magnification. At each experimental point, control cultures with normal bone marrow and spleen cells were included. The CFU-C number was calculated from the pooled results of two different cell numbers plated. The number of colonies per dish varied about 10% of the mean.

6. CFU-E Experiments

The method described by Iscove and Sieber [12] was used. We mixed 0.8% methylcellulose, 30% fetal calf serum, erythropoietin (Ep) step III (Connaught Lab.), 0.2–0.4 U/ml (depending on the optimal activity of the batch), α-thioglycerol at a final concentration of 10^{-4} *M*, and bone marrow or spleen cells in α medium. Four parallel dishes containing 1 ml with 3×10^5 cells/ml were set up, and incubated for 48 h as for CFU-C. Erythroid colonies containing more than eight small cells were scored without staining at a magnification of 80. Cells from FV-P-infected animals were also incubated without addition of Ep. The number of colonies grown without Ep (CFU-EI) was correlated to the number grown in the presence of Ep. If the same number was counted the erythroid cell system was considered 100% Ep-independent.

7. Drug Treatment

Tilorone was dissolved in distilled water and the appropriate dose was injected in 0.2 ml per 20 g mouse body weight.

Results

I. Effect of 80 mg/kg Tilorone IP on Hemopoiesis in Normal Mice

CBA/Ca mice were studied 1–20 days after tilorone injection. The results are presented in Figs. 1 and 2. The pluripotent (CFU-S) and the granuloid committed (CFU-C) stem cells in the marrow were very much depressed 1 and 2 days after the drug; they began to regenerate at day 5, but control values were not reached by day 20. In the spleen the initial fall was as in the marrow, but then an overshoot was present in the CFU-C concentration (at days 5, 12, 15, and 20) and also in the concentration of CFU-S at day 12. The spleens of tilorone-treated mice were about 20% bigger than control spleens. The data indicate a partial shift of the hematopoietic stem cells to the spleen. The CFU-E growth in the marrow (Table 1) was very low at days 1 and 2 and had regenerated at days 5 and 12. In the spleen of these CBA/Ca mice there is hardly any CFU-E growth, and therefore additional experiments were performed with DBA/2 mice. Their results confirmed the data obtained in the bone marrow. The initial fall in the CFU-E concentration was also seen in the spleen and followed by an overshoot between days 3 and 7. These marked changes in the different stem cell pools were not followed by marked alterations in the recognizable cell pools in the bone marrow smears, the total number of erythroblasts was even slightly increased. Slight reticulocytopenia in the peripheral blood was seen at day 2. Most remarkable, however, was the decrease in the number of lymphocytes per femur from days 1–10. In control animals about 6×10^5 lymphocytes were found, in mice treated with tilorone this number did not exceed 2.4×10^5 cells during this period (Table 2).

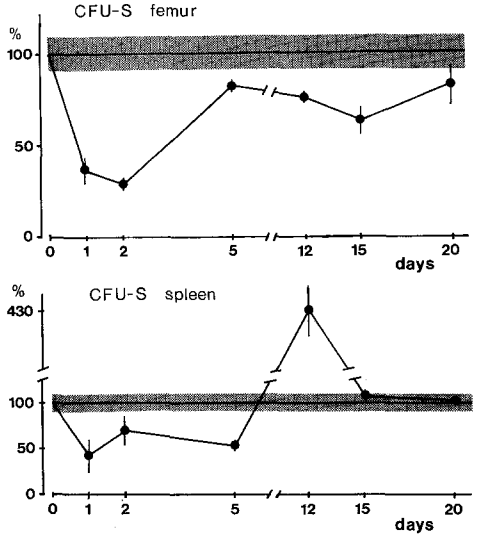

Fig. 1. Pluripotent stem cells (CFU-S) in femur and spleen of CBA/Ca mice treated with 80 mg tilorone/kg IP at day 0. Absolute numbers of CFU-S in daily controls varied between 2,400 and 2,600 per femur, and 10 and 18 per 10^7 cells in the spleen. Mean values ± SEM

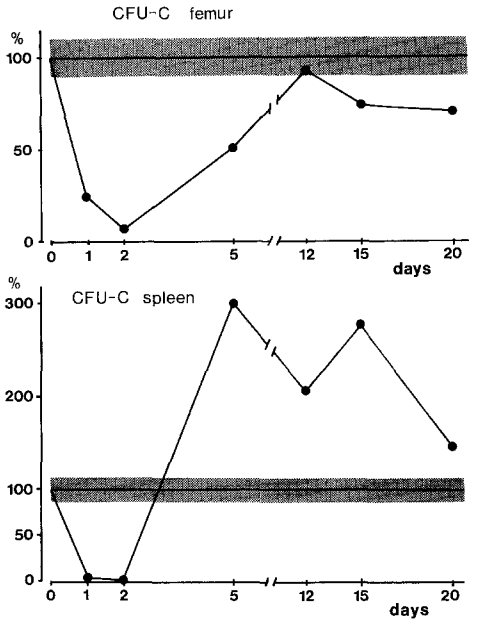

Fig. 2. Granuloid committed stem cells (CFU-C) in femur and spleen of CBA/Ca mice treated with 80 mg/kg tilorone IP at day 0. Absolute numbers of CFU-C in daily controls varied between 6,000 and 7,500 per femur, and 23 and 33 per 10^7 cells in the spleen. Mean values ± SEM

Table 1. Effect of tilorone[a] on CFU-E in the femur

Days after treatment	CFU-E/10^5	CFU-E per femur
Control	80[b]	12,900
Day 1	1	500
Control	110	16,300
Day 2	2	1,000
Control	98	14,000
Day 5	160	18,800
Day 12	96	16,200

[a] CBA/Ca mice received 80 mg/kg IP
[b] The SEM in all CFU-E cultures varied between 5% and 10%

II. Effect of Tilorone in FV-P-infected Mice

a) Treatment Before Virus Infection. Tilorone (80 mg/kg IP) was given 1 day before infection and the spleen weight and erythroid colony growth of bone marrow and spleen cells with and without addition of Ep were determined at days 6, 8, and 12. At day 8 after infection there was a small difference in the spleen weight and the number of reticulocytes was also reduced in the pretreated group. The number of Ep-independent CFU-E in the bone marrow was about 20% of the total number of CFU-E present (20 of 97) and also below the control value of about 80%

Table 2. Effect of tilorone[a] on the bone marrow

Days after treatment	Cells/femur ($\times 10^6$)	Differential in %				
		M_1–M_4	M_5–M_8	E_1–E_5	Lymphocytes	Others
1	16.9	10	37	28	19	1
2	14.8	12	36	28	21	1
5	12.3	9	38	34	16	1
7	11.8	9	47	34	7	1
10	15.8	9	43	28	15	1
Control	17.0	8	28	19	41	1

[a] CBA/Ca mice received 80 mg/kg IP

Table 3. Effect of tilorone treatment[a] on Friend leukemia development

		Spleen weight (mg)	Reticulocytes ‰	CFU-E per 10^5 cells			
				Bone marrow		Spleen	
				− Ep	+ Ep	− Ep	+ Ep
Day 5	Controls	104 ± 24	60 ± 10	0	170	0	70
	FV-P	151 ± 21	42 ± 14	6	50	60	85
	Tilorone + FV-P	157 ± 14	42 ± 13	2	105	50	81
Day 8	Controls	104 ± 24	60 ± 10	0	107	0	55
	FV-P	761 ± 121	172 ± 35	205	255	558	707
	Tilorone + FV-P	588 ± 109	73 ± 24	20	97	556	584
Day 12	Controls	104 ± 24	60 ± 10	0	93	0	55
	FV-P	1,716 ± 98	307 ± 29	46	51	235	235
	Tilorone + FV-P	1,781 ± 224	371 ± 18	34	33	241	253

[a] DBA/2 mice received 80 mg/kg IP, 1 day before FV-P injection

Table 4. Effect of tilorone treatment[a] on Friend leukemia development

		Spleen weight (mg)	CFU-E per 10^5 cells			
			Bone marrow		Spleen	
			− Ep	+ Ep	− Ep	+ Ep
Day 4	FV-P control	383	9	122	0	297
	FV-P + tilorone	386	10	111	909	897
Day 7	FV-P control	1,397	119	216	439	430
	FV-P + tilorone	1,614	304	276	645	671
Day 12	FV-P control	1,228	216	264	520	531
	FV-P + tilorone	1,718	224	232	568	600

[a] DBA/2 mice received 100 mg/kg SC on day 3 after FV-P injection

(205 of 255). At day 12 after virus infection no difference was seen between the normal FV-P-infected and the pretreated and then FV-P-infected group (Table 3).

b) Treatment After FV-P Infection. In the first series of experiments DBA/2 mice received 100 mg/kg tilorone SC 3 days after Friend virus infection, and the spleen weight and erythroid stem cells were studied at days 4, 7, and 12. The results are given in Table 4.

Control animals showed the normal replacement of normal Ep requiring CFU-E by Ep-independent ones. In the spleen all colonies became Ep-independent at days 7 and 12, i.e., the number of colonies per dish was the same with or without addition of Ep. In the bone marrow the percentage of Ep-independent ones rose from about 7% at day 4 (9 of 122) to about 80% at day 12. This is in agreement with our previous observations [22]. After tilorone treatment at day 3, an increase in the CFU-E concentrations in the spleen was seen at days 4 and 7, and all CFU-E growth was Ep-independent as soon as day 4. In the marrow all erythroid colonies became Ep-independent at day 7 in the treated group, but not in controls. The spleen weight was a little higher in the treated group at day 7.

In another series tilorone 80 mg/kg IP, was given at days 3, 6, and 9 after FV-P infection, and groups of mice were studied at days 5, 8, and 12. The results of one of three similar experiments are presented in Table 5. As seen from the spleen weight increase, the number of reticulocytes and the hematocrit the development of Friend leukemia was slightly retarded by this regimen. The analysis of the CFU-E growth of these mice showed that this could have been caused by a somewhat slower transformation of the erythroid system in the marrow into Ep inde-

Table 5. Effect of tilorone treatment [a] on Friend leukemia development

		Spleen weight (mg)	Reticulocytes (‰)	Hematocrit (%)	Ep-independence of CFU-E	
					Bone marrow	Spleen
Controls (all)		104 ± 24	60 ± 10	49 ± 2	0	0
Day 5	FV-P	151 ± 21	42 ± 14	51 ± 1	10%	70%
	FV-P + tilorone	156 ± 27	24 ± 2	49 ± 2	4%	60%
Day 8	FV-P	761 ± 121	172 ± 35	54 ± 3	80%	79%
	FV-P + tilorone	539 ± 198	90 ± 50	50 ± 1	68%	94%
Day 12	FV-P	1,716 ± 98	307 ± 29	64 ± 2	91%	100%
	FV-P + tilorone	1,681 ± 299	265 ± 36	57 ± 2	65%	100%

[a] DBA/2 mice received 80 mg/kg IP on days 3, 6, and 9 after FV-P injection

Table 6. Effect of tilorone on CFU-E growth in vitro

Tilorone (μg/ml)	CFU-E per 10^5 cells	
	Bone marrow	Spleen
0	309 ± 40[a]	179 ± 24[a]
0.01	300 ± 28	159 ± 23
0.05	321 ± 21	151 ± 17
0.08	327 ± 36	112 ± 16
0.1	321 ± 32	111 ± 7
0.5	270 ± 21	93 ± 12
0.8	213 ± 18	56 ± 8
1.0	91 ± 16	10
5.0	4	0
8.0	0	0
10.0	0	0

[a] Values are the mean of four dishes ± 1 SEM

pendence. To present the data more clearly only the fraction of CFU-E growing without Ep from the whole CFU-E compartment (with Ep) is given in percentage form in Table 5. The total number of CFU-E per femur was about the same in controls and tilorone-treated animals.

III. Effects of Tilorone on Erythroid Colony Growth in vitro

CFU-E colony growth from bone marrow of normal mice and CFU-EI colony growth (without Ep) from mice 14 days after FV-P infection was studied with in vitro addition of tilorone to the culture medium. As seen in Table 6 for normal CFU-E growth, 50% inhibition was reached at concentrations between 0.8 and 1.0 μg/ml and there was no difference in the CFU-EI cultures.

Discussion

The experiments described here clearly showed that tilorone has a cytotoxic effect on hematopoietic cells. Erythroid committed stem cells CFU-E, but also CFU-C and CFU-S, were markedly depressed after drug administration to normal mice. As seen after most toxic drugs in mice, the regeneration of committed stem cells, CFU-C and CFU-E, is accompanied by an overshoot in their concentrations in the spleen. The toxicity was not seen in the bone marrow smears, except that the number of bone marrow lymphocytes was reduced, and this may also explain the previous statement [32] that tilorone had no marrow-depressive effect in man in a study not including any stem cell studies.

In Friend leukemia only small effects were observed. Reports in the literature that the treatment of mice before virus infection would retard the development of the splenomegaly were confirmed. Repeated treatment after virus infection did slightly reduce the spleen weight compared with control animals, but the specific alteration in mice infected with FV-P, the development of Ep-independent erythropoiesis in bone marrow and spleen, replacing normal Ep-dependent erythropoiesis [22], remained unaffected. These effects of the drug seem to reflect a toxic action at the stem cell level with no influence on the virus-induced transformation into Ep-independence. In in vitro experiments normal CFU-E and CFU-EI from FV-P-infected animals had the same sensitivity to tilorone.

The depression of hematopoietic stem cells by the interferon inducer tilorone is in good agreement with a series of observations of cytotoxic effects of other inducers like Poly I Poly C [18], infection of mice with the acute lymphocytic choriomeningitis virus [3] or interferon itself [9, 23]. It has even been argued that the growth inhibitory factor, as seen in many other

experimental systems [4], might be identical with the antiviral factor [17].

From our experiments presented here it has to be concluded that the retardation of the spleen weight increase in mice infected with the Friend leukemia virus is mainly attributable to a general cytotoxic effect of tilorone on hematopoietic stem cells and at least in our tests no specific effect on the most characteristic virus-induced change, the transformation into Ep independence was seen, as might have been expected as a consequence of the inhibition of reverse transcriptase [27].

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