[CANCER RESEARCH 33, 2161-2165, September 1973]

Decrease in Oncogenic Potential of L1210 Leukemia by Triazenes[1]

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SUMMARY

Three or four lines each of L1210 were treated for 10 to 15 transplant generations with 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide, 1-phenyl-3,3-dimethyltriazene, and 1-phenyl-3-monomethyltriazene. With increasing numbers of maintenance generations, there was a marked increase in the survival times of the untreated control mice with all three compounds. In subsequent untreated transfer generations, most of the sublines did not take and the others retained reduced growth rates. X-irradiation, or treatment with the triazenes which severely depressed the white blood cell count, decreased the median survival times of the mice bearing the altered sublines but not to the level of the parent L1210. Transplantation into hamsters showed that all triazene-altered lines had less oncogenic potential than the parent line L1210. The results of the present experiments do not exclude changes in antigenicity but the heterotransplantation experiments show clearly that the altered lines lost oncogenic potential.

INTRODUCTION

In the continuing studies on drug resistance with murine leukemias in this laboratory, lines with greater response to chemotherapeutic agents than the parent lines (collateral sensitivity) were occasionally observed. Previously, we reported on the collateral sensitivity of a few of our long-established drug-resistant lines (13). We suggested that it is caused by antigenic change and/or a decrease in oncogenic potential of the cells.

Because of the likely importance of changes in antigenicity and malignancy with respect to a complete control of cancer we investigated the 3 triazenes DIC,[2] PDT, and PMT. Interest in triazenes was stimulated by reports in the literature. Clarke *et al.* (4) noted in 1955 that Sarcoma 180 failed to resume its usual rapid growth if pieces from tumor treated with PDT were implanted into normal mice. Bonmassar *et al.* (2) demonstrated that, after 4 different L1210 lines had been treated for several generations with DIC, a marked increase in the MST's of the untreated control mice was observed.

[1] Supported in part by Grant CA 08748 from the National Cancer Institute, Grant IC-66N from the American Cancer Society, and by the Elsa U. Pardee Foundation.

[2] The abbreviations used are: DIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; PDT, 1-phenyl-3,3-dimethyltriazene; PMT, 1-phenyl-3-monomethyltriazene; MST, median survival time.

Received April 24, 1973; accepted June 4, 1973.

MATERIALS AND METHODS

C57BL/6 × DBA/2 F_1 mice (here after called $BD2F_1$) 6 to 10 weeks old, obtained from A. R. Schmidt Co., Madison, Wis., were used. All groups consisted of 5 or 10 male mice.

For the development of the triazene-treated lines 1 million L1210 cells were injected i.p. Treatment consisted of DIC (100 mg/kg), PDT (50 mg/kg), or PMT (30 mg/kg) in 5 to 6 daily i.p. injections for 10 or 15 transplant generations. In each generation 1 million ascites cells obtained from the treated mice were implanted i.p. into each of 2 groups of mice: untreated and treated groups. Afterward the lines were maintained without treatment.

DIC, shortly before use, was suspended in 0.5% carboxymethyl cellulose in dark bottles. PDT and PMT were each dissolved in sesame oil and stored frozen at about −20°. The daily dose was contained in 0.1 ml of sesame oil per 20 g of mouse. DIC was supplied from the Drug Development Branch, Drug Research and Development, Chemotherapy, National Cancer Institute, Bethesda, Md. PDT was obtained from Merck and Co., Rahway, N. J. PMT was synthesized by us as previously described (5, 7). Melting point (37°) and absorption (maximum, 273 nm in ethanol) agree with the data of Preussmann and von Hodenberg (12).

The X-irradiated mice received 400 R total-body irradiation 24 hr prior to i.p. injection of the tumor cells. The factors were: 200 kVP; 9 ma; 0.4 mm copper filter; rate, 57 R/min.

Male golden hamsters (Chick Line, Newfield, N. J.) weighing 80 to 120 g were used for the heterotransplantation experiment. They received total body X-irradiation of 600 R 24 hr prior to s.c. injection of 10 million cells into the right axillary region. Twelve daily i.p. injections of streptomycin sulfate (100 mg/kg) were given starting on the day of irradiation.

Samples for blood counts were taken by orbital bleeding. For the determination of the growth rates, 50 male $BD2F_1$ mice were implanted i.p. with 1 million L1210 cells and 50 mice with an L1210 subline altered by treatment with PDT. Beginning on the 3rd day after implantation, 5 mice of each group were sacrificed on the specified days and the total i.p. cell counts were determined.

RESULTS

The effect of DIC on L1210 in the 4 treated groups of the 1st generation represents the antileukemic effect of DIC

(Table 1). The MST's were 11 or 12 days for the treated *versus* 8 days of the untreated mice. After several treatment generations, the MST's of the untreated control mice increased whereas the DIC-treated mice died at about the 12th day with generalized leukemia. After 15 treatment generations, the MST's of the untreated controls increased to 13, 23, and, in 2 lines, to more than 30 days. In subsequent untreated generations, 3 lines failed to produce lethal leukemia, and the MST of the 4th line after 50 generations (about 500 days) was 18 days.

The results after treatment with PDT and PMT were similar to those obtained with DIC (Table 1). All 3 PMT lines and 2 of the 4 PDT lines lost their ability to cause leukemic deaths. The other 2 PDT lines after 50 untreated transfer generations, retained reduced growth rates.

Smaller doses of PDT had less effect on L1210 during 15 transplant generations, and the i.p. route was superior to p.o. administration (Table 2).

The effect of total-body X-irradiation of 400 R of $BD2F_1$ mice bearing triazene-altered sublines is shown in Table 3. The MST's of the irradiated groups were shortened. The range of the MST's of the irradiated mice injected with 1 million cells of the altered lines was from 9 to 15, and after injection of 1000 cells it was 13 to 18 days.

For the study of oncogenic potential 10 million leukemic cells were injected s.c. into X-irradiated and streptomycin-treated golden hamsters (Table 4). Death due to leukemia was determined by bioassay of hamster liver suspension in $BD2F_1$ mice and by histological evaluation. The MST's of the triazene-altered lines ranged from 9 to more than 20 days, whereas the MST of the original L1210 line was only 6 days.

As shown in Table 1, mice bearing the triazene-treated leukemias and also treated with the triazenes died much earlier of leukemia than the corresponding untreated control groups of mice. This suggests that the shorter survival time of the treated mice was a consequence of the immunosuppressant activity of these drugs. Certain blood

Table 1

Development of triazene-altered L1210 lines

One million L1210 cells were injected i.p. and serially transplanted. Treatment consisted of 5 to 6 daily i.p. injections. One million cells from the treated mice were used for the untreated and treated groups of the next generation. Five $BD2F_1$ mice/group.

Transfer	MST (days)							
	Line 1		Line 2		Line 3		Line 4	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
				DIC, 100 mg/kg				
Treated								
1	8	12	8	12	8	11	8	12
5	14	12	14	11	19	15	17	13
10	15	12	12	12	>30	13	>30	13
15	23	15	13	11	>30	13	>30	13
Untreated								
4	NG[a]		11		>30		22	
6			15		26		25	
12			NG		28		24	
20					>30		14	
42					NG		15	
50							18	
				PDT, 50 mg/kg				
Treated								
1	8	12	8	12	8	12	8	12
5	22	11	13	12	15	11	21	11
10	29	12	20	12	>30	12	>30	14
15	>30	12	>30	14	>30	12	>30	16
Untreated								
3	>30		>30		>30		NG	
5	NG		>30		>30			
6			>30		28			
20			23		19			
50			18		27			
				PMT, 30 mg/kg				
Treated								
1	8	12	8	12	8	11		
5	30	12	30	12	17	12		
8	NG	NG	>30	19	17	14		
9			NG	NG	19	17		
10					18	13		
Untreated								
3					NG			

[a] NG, no growth.

Table 2

Effect of dose and route of treatment with PDT on serially transplanted L1210

One million L1210 cells were injected i.p. and 1 million cells from the treated mice were used for the untreated and treated groups of the next generation. Treatment consisted of single or 6 daily applications. Five $BD2F_1$ mice/group.

Generation	MST (days)									
	i.p.				p.o.				s.c.,	
	150 mg/kg (1 dose)		25 mg/kg (6 doses)		150 mg/kg (1 dose)		50 mg/kg (6 doses)		25 mg/kg (6 doses)	
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated	Untreated	Treated
Treated										
1	8	13	8	9	8	10	8	10	8	9
5	13	12	13	12	13	11	10	11	10	10
10	18	16	10	9	10	10	10	9	11	10
15	24	13	11	10	11	10	11	10	10	10
Untreated										
5	21		10		10		10		10	
10	20		10		10		10		10	
15	18		10		9		9		11	

Table 3

MST's of L1210 lines in normal and X-irradiated mice

Mice received 400 R total-body X-irradiation 24 hr prior to injection of cells.

	1,000,000 cells i.p.		1,000 cells i.p.	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
L1210	8	7	11	11
L1210/DIC 2[a]	11	9	17	15
L1210/DIC 3[a]	>30	15	>30	18
L1210/DIC 4[a]	22	10	>30	15
L1210/PDT 2[b]	>30	14	>30	16
L1210/PDT 3[b]	>30	14	>30	17
L1210/PMT 1[c]	20	13	23	17
L1210/PMT 2[c]	13	12	15	14
L1210/PMT 3[c]	14	12	16	13

[a] Untreated Transfer Generation 4, Table 1.

[b] Untreated Transfer Generation 5, Table 1.

[c] Untreated Transfer Generation 7 of 3 L1210 lines treated for 10 generations with PMT, 15 mg/kg, for 6 days.

Table 4

Heterologous transplantation of L1210 lines into golden hamsters

Hamsters received 600 R total-body X-irradiation 24 hr prior to s.c. injection of 10 million cells into the axillary region. Twelve daily i.p. injections of streptomycin sulfate, 100 mg/kg, were given starting on the day of irradiation.

Line	No. of hamsters	Leukemic deaths	MST (days)	20-day survivors
L1210	7	7	6	0
L1210/DIC 2[a]	7	7	9	0
L1210/DIC 3[a]	7	1	>20	5
L1210/DIC 4[a]	7	7	12	0
L1210/PDT 2[a]	7	5	14	1
L1210/PDT 3[a]	7	1	>20	5
L1210/PMT 1[b]	6	0	>20	4
L1210/PMT 2[b]	7	6	11	0
L1210/PMT 3[b]	6	6	9	0
Control	5	0	>20	4
Control, no streptomycin	5	0	8[c]	0

[a] Untreated Transfer Generation 6, Table 1.

[b] Untreated Transfer Generation 9 of 3 L1210 lines treated for 10 generations with PMT, 15 mg/kg, for 6 days.

[c] Death due to enteritis.

studies with the single 10% lethal doses of PDT and PMT in tumor-free mice were done to investigate their immunosuppressing activity (Table 5). There was essentially no difference in the degree of white blood cell depression caused by each drug. Both caused lasting and severe depression of granulocytes and agranulocytes. On Day 14 the counts remained low, and by Day 21 the counts had returned to about one-half of normal.

The marked increase in survival time of the untreated mice carrying the altered sublines over mice bearing the parent line L1210 could be due to dissimilarities in growth rate. As Table 6 shows, the growth rates of L1210 and an L1210/PDT line varied markedly. The total i.p. cell count on Day 6 for L1210 was 654 million, and for L1210/PDT 2 it was only 118 million. The greatest count for the L1210/PDT line was 905 million on Day 14. Plotting these data on semilog paper reveal that the doubling time for L1210 is about 12 hr and for L1210/PDT 2 it is 24 hr.

DISCUSSION

With increasing generations of treatment of L1210 leukemia with DIC, the untreated control leukemic animals exhibited a marked increase in survival time. This finding confirms the results obtained by Bonmassar *et al.* (2), whose L1210 sublines had an even more pronounced increase in survival time. Reasoning from the structure of DIC, we considered the triazene group as the active component. According to the work of Skibba *et al.* (14), demethylation appears to be a major metabolic pathway of DIC in rats and man. This is similar to the mechanism of carcinogenesis by PDT proposed by Preussmann *et al.* (11). They suggest demethylation of PDT to PMT which then by forming methyldiazohydroxide yields the carbonium ion. PMT tested in rats proved to be a proximate carcinogen (11). The

Table 5

Effect of single 10% lethal doses (i.p.) of PDT and PMT on the white blood cells of normal BD2F$_1$ mice

Five mice, selected at random, were killed on each day listed.

Day after injection	Wt change (g), Day 7	WBC (× 10^3/cu mm)			Spleen wt (mg)
		Total count	Granulocytes	Agranulocytes	
		Controls			
		8.3 ± 1.6[a]	2.1 ± 0.8	6.2 ± 1.1	80 ± 9
		PDT, 150 mg/kg			
1	−0.4	1.2 ± 0.5	0.6 ± 0.4	0.6 ± 0.2	58 ± 7
7	−3.1	1.9 ± 0.5	0.9 ± 0.4	1.0 ± 0.3	66 ± 25
14	−1.4	2.0 ± 1.0	0.7 ± 0.3	1.3 ± 0.9	90 ± 32
21	−0.5	4.6 ± 1.4	1.1 ± 0.7	3.5 ± 0.9	92 ± 18
		PMT, 90 mg/kg			
1	−0.2	1.3 ± 0.8	0.7 ± 0.6	0.6 ± 0.2	81 ± 23
7	−3.6	2.0 ± 0.8	1.1 ± 0.7	0.9 ± 0.3	46 ± 17
14	−2.1	2.6 ± 0.9	1.2 ± 0.2	1.4 ± 0.9	43 ± 8
21	−2.6	4.9 ± 1.2	1.0 ± 0.5	3.9 ± 1.1	65 ± 23

[a] Average ± S.D.

Table 6

Growth of leukemia L1210 and L1210/PDT 2[a] in BD2F$_1$ mice

One million cells were injected i.p. on Day 0.

Day	L1210 (million cells)	L1210/PDT 2 (million cells)
3	29 ± 11[b]	14 ± 7
4	111 ± 37	28 ± 14
5	432 ± 130	51 ± 29
6	654 ± 165	118 ± 50
7	410 ± 153	226 ± 133
14		905 ± 250
21		222 ± 295

[a] Untreated Transfer Generation 4, Table 1.
[b] Average of 5 mice ± S.D.

increase of the MST's of the untreated leukemic controls with increasing generations of treatment with DIC, as well as with PDT and PMT, clearly implicates the triazene group as the active component.

The difference in growth rate, as determined from the total i.p. cell counts, between L1210 and the altered lines was also apparent in their MST's. However, this great difference might be less real because the percentage of dead and dying cells is not indicated in these counts.

Preirradiation decreased the MST's of mice bearing the altered lines but not to the level of the parent L1210 line. Also treatment with the triazenes caused a decrease in survival time of mice bearing the altered lines as compared to the corresponding untreated mice. The immunosuppressant activity of these triazenes on the host was demonstrated by their severe effect on depression of the white blood cell counts.

The differences in MST between the irradiated and triazene-treated groups on one hand and the nonirradiated and nontreated groups on the other can be attributed either to different immunogenicity of the leukemic cells or to differences in the sensitivity of the cells to the response of the host, *i.e.*, oncogenic potential.

The results of the heterologous transplantation experiments in hamsters show that all triazene-altered lines had less oncogenic potential than the parent L1210 line, but they do not exclude changes in the antigenicity of these altered leukemia cells.

Kitano *et al.* (6) and Mihich (8) reported that a subline of L1210 leukemia resistant to methylglyoxal-bis(guanylhydrazone) was more antigenic for the DBA/2 host than the original sensitive line. Nicolin *et al.* (10) found that various drug-resistant sublines of L1210 were antigenically altered.

Relevant to this report are especially the studies of Bonmassar *et al.* (2, 3) on DIC-treated L1210 sublines. First (2) they favored the hypothesis that the development of their leukemia lines with increased survival time was due to the selection of antigenic variants, but later on the basis of additional studies (3) they suggested that DIC could activate a latent virus or induce somatic mutation at the level of histocompatibility antigens.

The results of the present experiment do not exclude changes in immunogenicity, but the heterologous transplantation experiments clearly demonstrate that the altered L1210 lines lost oncogenic potential. The same conclusions were arrived at previously with several resistant leukemia lines with pronounced collateral sensitivity (13). The mechanisms by which treatment with these triazenes causes reduced oncogenic potential and changes in antigenicity are unknown at present. The fact that some lines retained reduced growth potential after 50 transplant generations without treatment suggest that these changes are hereditarily determined.

Whether antigenic change and decrease in malignancy are incidentally or intrinsically associated is also unknown. It is relevant that tumor cell lines kept in culture for long times frequently lose malignant properties. Biedler and Riehm (1) demonstrated recently that actinomycin D- and daunomycin-resistant Chinese hamster cell lines had markedly reduced heterotransplantability; and Mondal *et al.* (9) obtained variants of decreased malignancy and antigenicity from clones transformed *in vitro* by methylcholanthrene.

ACKNOWLEDGMENTS

We thank Peter Tiffany and Barbara Bennett for expert technical assistance and Dr. A. Giner-Sorolla and Dr. B. Otter for advice in the preparation of PMT.

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