Inhibition of Ascites Cell Growth by Combinations of 6-Thioguanine and Azaserine*

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Azaserine has been shown to be a potent inhibitor of *de novo* purine synthesis in both solid tumors (1, 13) and ascites cells (5, 6, 10). The duration of inhibition has been correlated with increases in the survival time of tumor-bearing hosts (7, 8). During inhibition of *de novo* purine synthesis by azaserine, the ascites cells maintain the ability to utilize preformed purines and presumably survive through use of this mechanism (9, 12). An attempt was made to find an agent which would inhibit the utilization of preformed purines, thus producing a concurrent blockage of two alternate pathways, when combined with azaserine. 6-Thioguanine (3), an inhibitor of both animal neoplasms (2) and human leukemia (4), was found to inhibit guanine-8-C^{14} incorporation into nucleic acid guanine of Ehrlich ascites cells (11).

Tarnowski and Stock (14) have reported that, among a number of combinations, that of azaserine and thioguanine was synergistic in inhibiting the RC mammary carcinoma. No synergistic response was found when the mouse mammary carcinoma S-790 was used.

In these experiments the two drugs were used in various combinations to determine the effects of concurrent inhibition of two alternate pathways of purine biosynthesis on a spectrum of ascites tumor cells. The combination of antimetabolites was exceptionally effective against the Ehrlich ascites carcinoma, the TA3 ascites carcinoma, and the Sarcoma 180 ascites. The Mecca lymphosarcoma in solid or ascites form and the 6C3HED ascites were found to be relatively resistant.

MATERIALS AND METHODS

Five mouse tumors were employed in these experiments; they were the Ehrlich ascites carcinoma (hypertetraploid line) and Sarcoma 180 ascites in Swiss female mice,[1] the Mecca lymphosarcoma ascites[2] in AKD_2F_1 female mice, the TA3 ascites carcinoma in CAF_1 male mice, and the 6C3HED ascites lymphosarcoma in C3H female mice. Tumor transplantation was carried out by withdrawing ascites cells under light ether anesthesia with a #18 needle and hypodermic syringe. The cells were centrifuged for 2 minutes in a clinical centrifuge, the peritoneal fluid was decanted, and the cells were resuspended in isotonic saline. Swiss mice weighing 25–30 gm. were given inoculations of 10^7 Ehrlich or Sarcoma 180 cells; AKD_2F_1 mice weighing 16–20 gm. received an inoculation of 4×10^6 Mecca lymphosarcoma cells; CAF_1 mice weighing 18–25 gm. received 5×10^6 TA3 ascites cells; and C3H mice weighing 18–25 gm. received 10^7 6C3HED ascites cells. Mice were randomly distributed into groups and were maintained during the experiment on Purina Laboratory Chow pellets and water ad libitum. All experiments contained equivalent groups of control tumor-bearing animals receiving saline injections. Animals were weighed just prior to the first injection and again the day following termination of drug injections. The average weight change from the onset of therapy was recorded and used as an indication of drug toxicity.

The inhibitors were administered intraperitoneally, and therapy was initiated 24 hours after tumor implantation. This was continued for 6 consecutive days. Azaserine[3] was administered in isotonic saline in a volume of 0.25–0.50 ml. 6-Thioguanine[4] was dissolved in approximately two equivalents of sodium hydroxide and made up to volume with isotonic saline; the resulting solution of pH 7–8 was injected in a volume of 0.25–0.50 ml.

The survival time was used as the criterion of tumor inhibition. All animals free from tumor growth at 50 days were considered 50-day survivors in calculating the average survival time. These mice were routinely autopsied for solid tumors.

RESULTS AND DISCUSSION

The combination of azaserine plus thioguanine proved to be effective in inhibiting the Ehrlich ascites carcinoma. The results obtained with this tumor are summarized in Table 1. Thioguanine

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[1] Swiss mice were obtained from Taconic Farms, New York; the AKD_2F_1, CAF_1, and C3H mice from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

[2] Dr. Kanematsu Sugiura kindly supplied the Mecca lymphosarcoma as a subcutaneous implant; this was transformed into an ascites tumor.

[3] Azaserine was obtained through the courtesy of the Research Department of Parke, Davis & Co.

[4] An initial sample of 6-thioguanine was generously supplied by Dr. George Hitchings; subsequent supplies were synthesized in this laboratory.

was found to increase survival time of tumor-bearing mice to essentially the same extent when employed once daily at a level of 1.0 mg/kg for the standard 6-day therapy period used routinely in these experiments or when the dose was divided into two daily injections of 0.5 mg/kg. Decreasing the dose to 0.5 mg/kg once daily resulted in a concurrent decrease in survival time. Doses of 1.0 mg/kg and 1.5 mg/kg, injected twice daily, proved to be toxic to the tumor-bearing host. These animals lost weight throughout therapy, and the average survival time was the same as for saline-treated controls. No solid or ascites tumor masses were found; therefore, death animals, 50 per cent of the treated mice were found to be tumor-free. Two criteria were used to establish freedom from tumor growth. Surviving mice were kept for periods of 65–105 days. Any ascites cells surviving therapy should have been evident by this time. These animals were then routinely autopsied for the presence of solid tumors in the peritoneal cavity. A number of the autopsied animals had solid nodules along the intestinal wall. These were excised, fixed in formalin, sectioned, and upon microscopic examination were found to be areas of lymphoid hyperplasia.[5] Aggregations of lymph nodules which are visible grossly are known to occur in normal mice. Normal, healthy animals were kept under the same conditions for 50 days, and at this time they were autopsied. Similar areas of hyperplastic lymphoid tissue were found.

TABLE 1

EFFECTS OF DRUG THERAPY ON SURVIVAL TIME OF EHRLICH ASCITES TUMOR-BEARING MICE

Dose Mg/kg × daily dosage‡ Azaserine	Thioguanine	Average survival (days)	Animals free from tumor growth*/ Total no. animals	Av. wt. change† (gm.)
0	0	11.4±2.1§	0/20	+ 5.9
0	1.0×1	21.8±7.4	0/20	+ 9.1
0	0.5×1	15.1±2.5	0/20	+10.3
0	1.5×2	12.8±2.0#	0/5	− 1.4
0	1.0×2	10.8±1.0#	0/5	− 1.0
0	0.5×2	21.5±7.8	2/20	+ 5.2
0.2×2	0	20.4±2.1	0/14	+ 3.2
0.3×2	1.5×2	11.8±0.8#	1/5	− 3.4
0.3×2	1.0×2	9.6±1.4#	0/5	− 5.0
0.2×2	0.5×2	42.6±8.8	10/20	+ 1.3
0.2×2	1.0×1	38.8±9.4	4/20	− 0.3

* All mice free from tumor growth calculated as 50-day survivors.

† Average weight change at termination of drug treatment.

‡ Treated for 6 days, beginning 24 hours after tumor transplantation, with combination treatments given simultaneously.

§ Average deviation from mean.

Animals maintained on streptomycin-HCl drinking water (36 μg/ml) throughout experiment. Saline-treated controls receiving streptomycin-HCl drinking water had the same survival time as control animals receiving no streptomycin. These groups were, therefore, averaged together.

was adjudged to be due to toxicity. At these higher levels of thioguanine, the treated animals were maintained throughout their life-span on drinking water containing streptomycin to lessen the possibility of complication by infection.

Azaserine was administered in the dosage schedule previously found to be correlated with duration of inhibition of *de novo* purine synthesis (8, 9). This was employed as two daily doses administered 12 hours apart and was combined with thioguanine in this dose schedule. Injection of 0.2 mg/kg of azaserine twice daily produced a doubling of the survival time of Ehrlich ascites-bearing mice. The combination of 0.3 mg/kg of azaserine twice daily plus 1.0 mg/kg of thioguanine twice daily was toxic. An effective combination consisted of two daily doses of 0.2 mg/kg of azaserine and 0.5 mg/kg of thioguanine. When this dosage schedule was followed for groups of tumor-bearing

Combination therapy which employed a single daily injection of 1.0 mg/kg of thioguanine with the 0.2 mg/kg of azaserine twice daily was much less effective. When this dose sequence was utilized, only 20 per cent of the mice were found to be tumor-free.

In all experiments, no indication of drug toxicity, as determined by loss of weight, was found with the effective combinations.

Table 2 shows the effects of simultaneous and alternate drug therapy on the survival time of Ehrlich ascites tumor-bearing mice. Animals on alternate therapy received 0.2 mg/kg of azaserine

[5] The authors are grateful to Dr. Anton Lindner for the histological examination.

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at approximately 8:00 A.M. and 0.5 mg/kg of thioguanine at 8:00 P.M. Simultaneous therapy consisted of 0.2 mg/kg of azaserine plus 0.5 mg/kg of thioguanine injected at 8:00 A.M. Treatment in both cases was for 6 consecutive days. An additive response was produced with alternate drug injections, while simultaneous treatment produced a marked synergistic response.

The results obtained with the Sarcoma 180 ascites are shown in Table 3; the results were similar to those seen with the Ehrlich carcinoma. The same degree of inhibition was produced by doses of thioguanine when administered either as 0.5 mg/kg twice daily or 1.0 mg/kg once daily. The combination of a twice-daily injection schedule of 0.5 mg/kg of thioguanine plus 0.2 mg/kg of azaserine again was the most effective one used; 60 per cent of the treated animals were free from tumor growth. One daily dose of 1.0 mg/kg of thioguanine plus the two daily injections of 0.2 mg/kg of azaserine resulted in 30 per cent of the mice being free from tumor growth.

To lessen the role played by immunological factors, three strain-specific tumors were included in the screening program. One of these was the TA 3 ascites carcinoma. This tumor was also found to

TABLE 2

EFFECTS OF SIMULTANEOUS AND ALTERNATE DRUG THERAPY ON SURVIVAL TIME OF EHRLICH ASCITES TUMOR-BEARING MICE

Dose Mg/kg × daily dosage‡ Azaserine	Thioguanine	Average survival (days)	Animals free from tumor growth*/ Total no. animals	Av. wt. change† (gm.)
0	0	9.3±1.6§	0/10	+4.0
0	0.5×1	14.4±2.5	0/10	+7.7
0.2×1	0	17.8±2.3	0/10	+4.8
0.2×1	0.5×1	21.7±2.6	0/20	+4.7
0.2×1 Alternate#	0.5×1	35.6±11.0	6/20	+1.7
Simultaneous#				

* All mice free from tumor growth calculated as 50-day survivors.

† Average weight change at termination of drug treatment.

‡ Treated for 6 days, beginning 24 hours after tumor transplantation.

§ Average deviation from mean.

Alternate therapy consisted of 0.2 mg/kg of azaserine at approximately 8:00 A.M. and 0.5 mg/kg of thioguanine at approximately 8:00 P.M.; simultaneous therapy consisted of 0.2 mg/kg of azaserine plus 0.5 mg/kg of thioguanine both injected at 8:00 A.M.

TABLE 3

EFFECTS OF DRUG THERAPY ON SURVIVAL TIME OF SARCOMA 180 AND TA3* ASCITES TUMOR-BEARING MICE

Tumor	Dose Mg/kg × daily dosage§ Azaserine	Thioguanine	Average survival (days)	Animals free from tumor growth†/ Total no. animals	Av. wt. change‡ (gm.)
Sarcoma 180	0	0	12.3± 2.0#	0/20	+ 5.0
	0	1.0×1	18.0± 3.5	1/20	+10.8
	0	0.5×2	19.6± 4.4	0/20	+ 8.4
	0.2×2	0	20.7± 3.2	0/15	+ 1.0
	0.2×2	0.5×2	43.0± 9.8	12/20	+ 0.4
	0.2×2	1.0×1	38.2±12.1	6/20	+ 1.2
TA3	0	0	17.2± 2.8	0/29	+ 1.4
	0	0.5×2	22.8± 8.8	1/19	− 1.7
	0.2×2	0	24.4± 5.6	0/18	− 0.9
	0.2×2	0.5×2	44.0± 5.7‖	9/39	− 2.0

* TA3 ascites tumor-bearing animals maintained on streptomycin-HCl drinking water (36 μg/ml) throughout experiment.

† All mice free from tumor growth calculated as 50-day survivors.

‡ Average weight change at termination of drug treatment.

§ Treated for 6 days, beginning 24 hours after tumor transplantation, with combination treatments given simultaneously.

Average deviation from mean.

‖ At 50 days fourteen mice had survived; of these, nine were tumor-free. All such animals were calculated as 50-day survivors.

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be susceptible to the combination therapy of two daily doses of 0.5 mg/kg of thioguanine and 0.2 mg/kg of azaserine, as shown in Table 3.

The other strain-specific ascites tumors used were the Mecca lymphosarcoma and the 6C3HED lymphosarcoma. These were quite resistant to the dose levels employed. Table 4 illustrates the refractoriness of these two tumors.

Resistance may be due to biochemical differences in the lymphosarcoma cells, or, since these cells are extremely invasive, the ascites cells may rapidly become disseminated throughout the host, and therapy at the low dose levels employed for the ascites test system would not be sufficient to prolong life to any appreciable extent. To test this possibility, the dose levels of thioguanine and azaserine were increased and used against both an intraperitoneal and a subcutaneous implant of the Mecca lymphosarcoma. The results in Table 4, Experiment 2, indicate that increasing the azaserine dosage to 2.0 mg/kg and the thioguanine level to 15.0 mg/kg, both administered for 3 consecutive days, did not produce any further prolongation of survival time. A dosage schedule which employed six daily treatments of 0.5 mg/kg of azaserine plus 2.0 mg/kg of thioguanine proved to be toxic. However, autopsy revealed that only two of the ten animals in this experiment had any grossly detectable ascites growth, indicating that some degree of inhibition of the Mecca lymphosarcoma cells had occurred.

TABLE 4

EFFECTS OF DRUG THERAPY ON SURVIVAL TIME OF MECCA LYMPHOSARCOMA AND 6C3HED LYMPHOSARCOMA TUMOR-BEARING MICE

Tumor	Tumor inoculation site	Dose Mg/kg × daily dosage Azaserine	Thioguanine	Average survival (days)	Animals free from tumor growth/ Total no. animals	Av. wt. change (gm.)*
Mecca	Exp. 1 Intraperitoneal implant†	0	0	11.0±1.0‡	0/20	+1.3
		0	1.0×1	10.9±0.6	0/19	+0.1
		0	0.5×2	10.5±1.0	0/20	+0.8
		0.2×2	0	11.5±0.8	0/20	+1.1
		0.2×2	0.5×2	13.0±0.6	0/20	+0.4
		0.2×2	1.0×1	12.7±0.6	0/20	+0.8
	Exp. 2 Intraperitoneal implant#	0	0	10.4±1.3	0/10	+1.1
		2.0×1	15.0×1	12.2±1.6	0/10	−4.5
		0.5×2	2.0×2	10.8±1.1	0/10	−5.6
	Subcutaneous implant‖	0	0	14.9±1.1	0/10	+1.2
		2.0×1	15.0×1	17.2±3.0	0/10	−2.6
6C3HED	Intraperitoneal implant†	0	0	15.1±2.7	0/11	+2.6
		0.2×2	0.5×2	16.3±1.3	0/11	−0.4

* Average weight change at termination of drug treatment.

† AKD_2F_1 mice received an inoculation of 4×10^6 Mecca lymphosarcoma cells; C3H mice received 10^7 6C3HED ascites cells. Treatment was for 6 days, beginning 24 hours after tumor transplantation, with combination treatments given simultaneously.

‡ Average deviation from mean.

Animals received an inoculation of 10^7 tumor cells; 2.0 mg/kg of azaserine plus 15.0 mg/kg of thioguanine was administered for 3 consecutive days, beginning 24 hours after tumor transplantation; 0.5 mg/kg of azaserine plus 2.0 mg/kg of thioguanine was administered for 6 consecutive days, beginning 24 hours after tumor implantation. Combination treatments were given simultaneously.

‖ Animals received an inoculation of 7×10^6 tumor cells.

SUMMARY

The Ehrlich ascites carcinoma, the Sarcoma 180 ascites, and the TA 3 ascites carcinoma were found to be sensitive to combinations of azaserine plus thioguanine. The Mecca lymphosarcoma in either solid or ascites form and the 6C3HED lymphosarcoma ascites proved to be relatively resistant. The most effective dose schedule tried was 0.2 mg/kg azaserine plus 0.5 mg/kg thioguanine, given twice daily, beginning 1 day after the tumors were transplanted. After 6 days of this therapy, 50 per cent of mice transplanted with Ehrlich ascites carcinoma, 60 per cent of mice transplanted with Sarcoma 180 ascites, and 23 per cent of mice transplanted with TA3 ascites carcinoma were found to be tumor-free.

Simultaneous therapy of 0.2 mg/kg of azaserine plus 0.5 mg/kg of thioguanine, both injected at approximately 8:00 A.M. daily for 6 days, was much more effective than alternate therapy, which consisted of 0.2 mg/kg of azaserine at approximately 8:00 A.M. and 0.5 mg/kg of thioguanine at

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approximately 8:00 P.M. for a total of 6 days. This indicates that, for maximum tumor inhibition, thioguanine and azaserine must be administered simultaneously, supporting the concept that the two drugs establish concurrent blocks of purine nucleotide synthesis.

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