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Spirogermanium: Effects on Hematopoietic Stem Cells and Survival of Normal and Tumor-Bearing Mice[1] (41627)

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Abstract. The effect of spirogermanium (SG) on hematopoietic stem cells, tumor burden, and survival times was investigated in C3H mice with transplanted mammary carcinoma. Compared to normal mice, the number of hematopoietic stem cells, or colony-forming units per spleen (CFU-S), was lower in the marrow of tumor-bearing mice. Spirogermanium at 15 and 30 mg/kg was not toxic to the normal hematopoietic cells in the marrow of either normal or tumor-bearing mice. In contrast to animals treated with cyclophosphamide, SG did not decrease the tumor growth rate or prolong the survival times of tumor-bearing C3H mice. Doses of 35–40 mg/kg SG did not prolong the survival times or decrease the tumor burden of AKR/J mice with a long-passaged lymphoma. These studies demonstrate that SG has minimal inhibitory effects to the marrow of normal mice and may promote the maintenance of normal marrow cells in tumor-bearing animals. However, in two different transplanted tumor cell lines, SG did not inhibit tumor growth or prolong host survival time.

A major limiting factor in the administration of chemotherapeutic agents is toxicity to normal hematopoietic cell populations (1, 2). Likewise, the presence of malignant cells has been associated with changes in normal hematopoietic stem cell proliferation and differentiation (3–8). Thus, to evaluate particular therapeutic strategies, it is important to know the effects of treatment on normal hematopoietic cells as related to the development and treatment of malignant tumor systems. Transplanted mouse tumor models have been successfully used for the preliminary screening of many cancer therapy protocols. With these tumor models, it is feasible to evaluate the effects of various treatments on the malignant cells as well as on the hematopoietic stem cell compartments (8–11).

Spirogermanium (SG) is a germanium-substituted azaspirane that has been shown to have antitumor activity on some types of rat and human neoplasias (12, 13). It appears to act on rapidly dividing cells by inhibiting protein synthesis (12). The limited hematopoietic toxicity of SG (13–15) warranted further studies to evaluate its usefulness in the treatment of both solid and disseminated malignancies.

In this report, the usefulness of SG in prolonging the survival times of tumor bearing animals was investigated both in mice with a transplanted AKR(Rb6.15)lAld lymphoma and animals with a transplanted C3H mammary carcinoma. Tumor burden and survival times were compared in mice injected with single or multiple doses of SG or cyclophosphamide. In addition, the effects of a single dose of SG on hematopoietic stem cell populations were investigated in non-tumor- and tumor-bearing mice.

Materials and Methods. *Mice.* Recipient and normal donor mice used in these studies were 8- to 16-week-old AKR/J, C3HeB/FeJ, or (C57Bl/6J × DBA/2)F_1 or B6D2F_1 females purchased from Jackson Laboratories, Bar Harbor, Maine. The tumor and drug-treated C3HeB/FeJ mice were housed in individual cages. Irradiated C3HeB/FeJ and all AKR/J mice were housed 5–10 per polypropylene cage. Irradiated mice were maintained in a laminar-flow hood until the time of sacrifice. All animals were allowed food (Purina Lab

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Chow) and HCl-acidified water (pH 2.4) *ad libitum.*

Transplanted tumor lines: C3H mammary carcinoma tumor line. This line originated as a mammary carcinoma in a C3H female mouse and has been maintained by transplantation into C3HeB/FeJ mice (7). Tumor cells were passaged by subcutaneous injections of 2×10^5 viable cells in 0.1 ml M199 medium into the left hind leg, distal to the popliteal lymph node. Within 14–19 days a tumor with a diameter of 4–5 mm (as measured using a vernier caliper) had developed, and at this time the drug treatments were started. Prior to each injection, the diameter of the tumor was measured, and the body weight and general health of the animal were noted.

AKR(Rb6.15)lAld lymphoma cell line. This cell line has been maintained by transplantation into normal AKR/J mice (10). Lymphoma cells were passaged by iv injections of 1×10^6 nucleated spleen cells. By Day 6, the recipient mice had enlarged spleens and extensive infiltration of the bone marrow, spleen, and lymph nodes with donor-derived lymphoma cells. The animals died within 7 ± 2 days after injection of the lymphoma cells. Chromosome markers (two metacentric chromosomes) present in the AKR(Rb6.15)lAld-derived lymphoma cells were used to follow the growth and distribution of the transplanted lymphoma cells in AKR/J mice (10).

Drug treatments. All drugs tested were diluted so that 0.01 ml solution contained the dose to be injected per gram of body weight. No single animal received more than 0.4 ml per injection. Cyclophosphamide (Mead-Johnson, Evansville, Ind.) was prepared in sterile distilled water and administered by intraperitoneal injections. Spirogermanium (kindly supplied by Dr. M. G. Mulinos, Unimed, Somerville, N.J.) was dissolved in sterile saline and within 30 min was administered by intravenous or intraperitoneal injection.

Preparation of cell suspensions. Mice used as cell donors were sacrificed by cervical dislocation, and the tissues for study were excised and weighed. Cells for injection were flushed from the tissues with Hank's balanced saline solution (HBSS) and dispersed through a 25-gauge needle. The cell suspensions were then diluted to the desired concentrations for injection in 0.2 ml HBSS. This volume was then injected into a caudal vein of each mouse. Quantitative nucleated cell counts were done on the bone marrow from the humeri of individual mice as described by Chervenick *et al.* (16). In each group, equal volumes of cell suspensions from quantitative marrow preparations of individual mice were pooled. These cell suspensions were then dilated, and cells equivalent to 0.01 humerus were injected into irradiated mice to assay for CFU-S.

Assays for CFU-S and spleen ^{59}Fe uptake. The irradiation source used in these studies was a ^{137}Cs irradiator (Model Mark I, J. L. Shepherd and Associates, Glendale, Calif.). The mice were exposed at a dose rate of approximately 120 rad/min.

The assay for 8-day colony-forming units on the spleen (CFU-S) was done as described by Till and McCullough (17). Seventeen hours before the mice were sacrificed, they were injected ip with 0.1 μCi ferrous citrate-^{59}Fe (Mallinkrodt Nuclear) diluted in 0.2 ml of 0.003 *M* citrate. The excised spleens were placed into individual vials of Bouin's fixative and counted in a well-typed scintillation counter (Nuclear Chicago). The mean percentage of injected ^{59}Fe uptake per spleen was calculated for each group of experimental mice, and the number of macroscopic, raised white nodules (colonies) was counted for each spleen.

Statistics. Since cell counts tend to be skewed (18), geometric means (i.e., logarithmic transformations) were used to obtain a more normal distribution of variables (19, 20). The weighted mean and standard errors were used to combine data from similar experiments (19). The Student *t* test was used to test for significant differences between groups of mice.

Results. *Effect of SG on survival of normal mice.* When normal C3HeB/FeJ mice were injected with 30 mg/kg SG, all of the animals ($n = 10$) had severe convulsions. Sixty percent of these animals died within 2 min after iv injection of the drug. Mice treated with 15 mg/kg SG also had convulsions immediately after the iv injection, however, within 24 hr the mice were active, eating, and drinking. In contrast to the C3HeB/FeJ mice, no apparent adverse effects were seen in $B6D2F_1$ mice administered 30 mg/kg by iv injection ($n = 11$).

Thus, the latter strain was used to assess the effects of a higher drug dosage on hematopoietic stem cells.

Effect of SG on hematopoietic stem cells in the marrow of normal and tumor-bearing mice. The number of colony-forming units per spleen (CFU-S) and the percentage ^{59}Fe uptake (a measure of erythropoiesis) were measured in the spleens of mice receiving marrow cells. These parameters were used to quantitate the effect of SG treatment on pluripotential hematopoietic stem cells from normal and tumor-bearing mice. The total number of nucleated cells per humerus was not significantly different ($P < 0.05$) in normal or tumor-bearing mice (Table I, B). However, when compared to normal mice, the number of CFU-S per humerus from tumor-bearing mice was significantly lower ($P < 0.01$). There was no apparent effect of 15 mg/kg SG on marrow cellularity in normal or tumor-bearing mice (Table 1, A and B). The mean number of CFU-S per 0.01 g humerus and the percentage ^{59}Fe uptake per spleen of marrow recipients were not significantly different ($P < 0.05$) between saline- or SG-treated mice. Even in the presence of a tumor, SG treatment did not further decrease the number of CFU-S per humerus (Table I, B).

The effect of a 30-mg/kg dose of SG on CFU-S was studied using $B6D2F_1$ strain of mice. After 4 days, the total number of nucleated cells per humerus was higher ($P < 0.05$) in the SG-treated animals than in the saline-injected mice (Table I, C). However, the number of CFU-S per humerus and percentage ^{59}Fe uptake per spleen were not significantly different ($P > 0.05$).

Comparison of SG and cyclophosphamide on tumor size and survival times of CeHeB/FeJ mice. Tumor size and survival times were compared in C3HeB/FeJ mice administered saline, cyclophosphamide, or SG. In nontreated mice, the mean diameter of the tumor increased from 4.1 ± 0.8 mm to 25.4 ± 2.6 mm (Fig. 1). Four weekly ip injections of 60 mg/kg cyclophosphamide significantly decreased the growth rate of the tumor compared to the nontreated mice. One week after the last injection (Week 3) of cyclophosphamide, the mean tumor size was 16.3 ± 2.8 mm with all of the animals still alive. The saline-injected mice died within 32 ± 4 days after start of the treatment regimen. Cyclophosphamide significantly prolonged the survival time of the animals to 46 ± 3 days. A similar treatment schedule with 15 mg/kg (ip) SG did not significantly decrease the growth rate of the tumor. At all times studied, the mean tumor diameter was approximately the same as seen in the nontreated animals. The mean survival times of the SG treated mice was 33 ± 5 days. Treatment with SG did not significantly prolong the survival times of the

TABLE I. EFFECT OF SPIROGERMANIUM ON HEMATOPOIETIC STEM CELLS IN MARROW OF NORMAL AND TUMOR-BEARING MICE

Mouse	Treatment[a]	Nucleated cells/ humerus of treated mice ($\times 10^3$)[b]	Number of colonies/ spleen (CFU-S) of recipients[c]	Percentage ^{59}Fe uptake/spleen of marrow recipients[d]
A. C3HeB/FeJ				
Normal	Saline	6111 ± 299	17 ± 2	4.0 ± 0.5
	SG-15 mg/kg	5402 ± 354	13 ± 2	4.1 ± 0.9
B. C3HeB/FeJ				
Normal	Saline	7012 ± 395	15 ± 1	2.5 ± 0.3
Tumor-bearing	Saline	7030 ± 217	10 ± 1	1.8 ± 0.2
Tumor-bearing	SG-15 mg/kg	6629 ± 541	9 ± 2	1.8 ± 0.3
C. $B6D2F_1$				
Normal	Saline	6436 ± 225	21 ± 1	4.9 ± 0.6
	SG-30 mg/kg	8149 ± 331	25 ± 1	4.1 ± 0.4

[a] Intravenous injections.

[b] Two (C3H) or 4 ($B6d2F_1$) days after treatment with saline or SG (geometric means ± SE).

[c,d] Number of macroscopic colonies (*c*) and percentage ^{59}Fe uptake/spleen (*d*) in 10–11 irradiated syngenic recipients from treated animals ($n = 5$) that received 1% of the total cell suspension (geometric means ± SE).

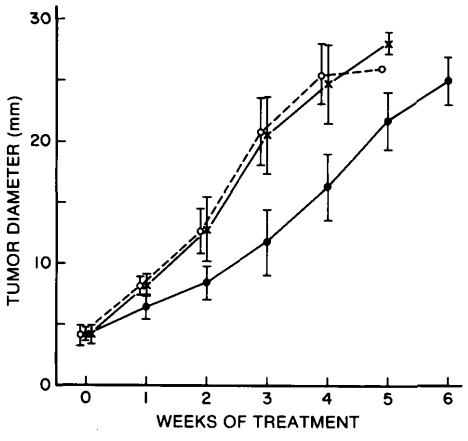

FIG. 1. Effect of spirogermanium and cyclophosphamide on tumor growth in C3HeB/FeJ mice. Drug treatments were initiated 15 days after sc injection of 2×10^5 tumor cells. Tumor diameter was measured weekly. Spirogermanium (15 mg/kg), cyclophosphamide (60 mg/kg), and saline were administered by intraperitoneal injections four times at weekly intervals starting at time zero. Data represents the mean ± SD for tumor size in surviving animals (n = 11–12/group at time zero).

tumor bearing animals. In addition, 50 mg/kg of SG administered ip twice a week was without effect in decreasing tumor burden or prolonging the survival times of the treated animals (data not shown).

Effect of SG on survival of AKR/J mice with transplanted lymphoma. SG was administered by ip injection to AKR/J mice at Day 3 of a long-passaged lymphoma. Injections of 35, 70, 100, or 140 mg/kg SG did not prolong the survival of these mice. In all groups, the mice (n = 10/group) died within 6 ± 1 days after the injection of lymphoma cells. This was the same as for the saline-injected mice with lymphomas.

Discussion. In this report, a C3H transplanted tumor model was used to evaluate the effects of SG on normal and malignant cell populations within the same animal. Alterations in hematopoietic stem cell and mature cell populations may occur due to the presence of malignant cells (3–6). Therefore, studies were done to compare the effects of SG on normal hematopoietic cell populations in normal and tumor-bearing mice. Spirogermanium did not appear to have any toxic effects on hematopoietic stem cells (CFU-S) in the marrow of normal or tumor-bearing mice. When compared to the control non-tumor-bearing C3HeB/FeJ mice, tumor-bearing animals showed a significant decrease in the number of CFU-S. After iv administration of 15 or 30 mg/kg of SG, there was no further decrease in the CFU-S of tumor-bearing mice.

The effects of single and multiple doses of SG and cyclophosphamide on prolonging survival time and decreasing tumor burden were compared in tumor-bearing C3HeB/FeJ. Cyclophosphamide is known to be effective in killing malignant cells in C3H and AKR transplanted-tumor models (21, 22). The rate of tumor growth was significantly decreased in cyclophosphamide-treated C3HeB/FeJ mice. Also, weekly injections of cyclophosphamide significantly prolonged the survival time of these animals. However, in mice administered weekly or biweekly doses of 15 mg/kg or 50 mg/kg SG, tumor growth and survival time were approximately the same as in the nontreated group of animals.

The effect of a single dose of SG or BCNU on prolonging survival time and decreasing tumor burden has been studied in AKR mice (10). In AKR mice with a transplanted AKR(Rb6.15)lAld lymphoma, it is possible to use chromosome analysis to differentiate between normal (i.e., host-type) and lymphoma (i.e., donor-type) metaphase cells (10). After treatment with BCNU, the total number of marrow cells was significantly decreased in mice with transplanted lymphoma. Both normal and lymphoma cell populations were decreased in the marrow of BCNU-treated mice. However, there appeared to be a preferential toxic effect on the proliferating lymphoma cell populations. In SG-treated animals while the percentage of lymphoma derived metaphase cells was also decreased, the total number was not affected. Thus, it is not unexpected that SG had no significant effect on prolonging the survival times of the lymphoma bearing mice. However, these results suggest a role for SG in the maintenance of normal marrow cells in tumor-bearing animals.

Spirogermanium has been shown to have antitumor activity in some types of rat and human neoplasias (12, 13). However, SG did not decrease tumor burden or prolong survival times in mice with a transplanted C3H mammary carcinoma or an AKR(Rb6.15)lAld long-passaged lymphoma (10). It has been demonstrated that SG is not effective in the treatment of certain types of transplanted murine tumors (12). One explanation that has

been proposed is that SG is bound to some component in mouse serum and is thus unable to interact with malignant cells (12). Our results may also be explained by the fact that, in contrast to spontaneous or first-passage tumor cells (23, 24), long-passaged malignant cells are not sensitive to the doses of SG currently employed. Even though SG did not show antitumor activity in these studies, data presented in this report with normal B6D2F$_1$ mice and earlier with lymphoma-bearing AKR mice (10) suggest that treatment with SG may stimulate the proliferation of some normal cell populations. An agent that is capable of promoting earlier regeneration of surviving normal cell populations may be beneficial when used in conjunction with chemotherapeutic protocols.

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