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Tilorone-Mediated Protection against Murine B16 Melanoma (41210)

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Abstract. C57B1/6 mice injected subcutaneously with B16 melanoma cells and treated intraperitoneally with 1 mg tilorone daily following tumor challenge demonstrated a reduction in tumor incidence and prolongation of survival in comparison with controls. While tilorone exhibited nonselective toxicity for B16 cells *in vitro*, it also induced macrophage-mediated inhibition of B16 tumor cell growth in culture. Thus, both cytotoxic effects and enhanced macrophage functions appear to have contributed to the reduced B16 melanoma growth observed here in tilorone-treated animals.

Tilorone is a low-molecular-weight compound with numerous biologic activities including interferon induction (1–3), antitumor activity (4–10), enhancement of antibody production (8, 11–13), suppression of certain cell-mediated immune responses (12, 13–15), and alteration of lymphoid cell populations in the blood and peripheral lymphoid tissues (13, 16, 17). Studies of the organ distributions of tilorone suggested that it might bind melanin since relatively high concentrations of tilorone were found to be associated with the pigmented tissues of the eye (18). In view of possible melanin binding by tilorone and also because of its reported antitumor activity for some tumors, it was of interest to examine the effects of this material on the growth of B16 murine melanoma. Tilorone treatment was found in this study to decrease tumor incidence and to prolong survival of B16 challenged mice.

Materials and Methods. *Animals.* Inbred C57B1/6 male mice from Jackson Laboratories (Bar Harbor, Maine) were used in this study.

Tumor. B16 melanoma cells were derived from a tumor-bearing mouse obtained from Jackson Laboratories (Bar Harbor, Maine) and adapted to tissue culture. Cell cultures were maintained in RPMI 1640 medium supplemented with L-glutamine, 5% fetal calf serum (Grand Island Biologicals, Grand Island, N.Y.) and gentamycin (Schering Corp., Kenilworth, N.J.) at 37° in 5% CO_2 in air. Monolayers of B16 cells for injection were thoroughly washed with Hanks' balanced salt solution, scraped with a rubber policeman, centrifuged, resuspended in serum-free RPMI 1640 medium, and viability was determined. Suspensions of tumor cells were injected sc in 0.1-ml volumes into groups of C57B1/6 mice. The mice weighed 18–20 g at the time of tumor cell injection. Dose–response studies demonstrated that 90–100% of animals given 10^4 B16 cells developed tumors and subsequently died.

Tilorone. Tilorone hydrochloride, 2,7-bis(2-(diethylamino)ethoxyfluoren-9-one, or [^{14}C]tilorone hydrochloride (gift of Merrell-National Laboratories, Division of Richardson-Merrell, Inc., Cincinnati, Ohio) was dissolved in sterile saline for injection purposes or in RPMI 1640 medium for *in vitro* studies.

Toxicity. In vitro toxic effects of tilorone for B16 melanoma cells were examined by the use of ^{51}Cr-labeled cells to evaluate direct toxicity and by the use of [^{3}H]-thymidine ([^{3}H]TdR) to measure growth inhibition. Fibroblasts derived from trypsinized C57B1/6 mouse fetal tissues were used in these studies for comparison with B16 cells. Labeling of cell suspensions with ^{51}Cr was accomplished by incubating 10^8 cells with 50 μCi sodium chromate (Amersham, Arlington Heights, Ill.) for 30 min at 37° followed by thorough washing. Cultures of 10^4 labeled cells in 1 ml volumes with and without various concentrations of tilorone were incubated

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and the amount of ^{51}Cr release was determined from the culture supernatants. The percentage release was determined:

$$\text{Percentage release} = \frac{\text{(cpm of supernatant from cells incubated with test material)} - \text{(cpm of supernatant from control cells)}}{\text{(cpm of supernatant obtained by lysis of control cells)}} \times 100.$$

Supernatants were counted in a Beckman 310 gamma counter.

Growth inhibition of B16 melanoma cells or C57B1/6 fibroblasts by various concentrations of tilorone was evaluated by culturing 10^3 cells per well of Microtest II plates (Falcon Plastics, Oxnard, Calif.) for 6 days. Cultures were pulsed 24 hr with 1 μCi [^{3}H]TdR (Research Products International, Elk Grove Village, Ill.) before harvest with a MASH II sample harvester (Microbiological Associates, Bethesda, Md.). Filters were counted in a Beckman LS-230 counter. The percentage growth inhibition was determined:

$$\text{Percentage growth inhibition} = \frac{\text{(cpm of control cultures)} - \text{(cpm of test cultures)}}{\text{(cpm of control cultures)}} \times 100.$$

All groups were prepared in triplicate, the results were averaged and standard error of the mean for each group was calculated.

Peritoneal exudate cells (PEC). PEC were harvested 5 days after the ip injection of 2 ml thioglycollate. Tilorone-treated PEC were obtained from similarly injected mice given 1 mg tilorone ip 24 hr before harvest of PEC. Harvested cells were incubated in tissue culture dishes and rinsed thoroughly after 1 hr to remove nonadherent cells. Plates were further incubated for 2 days to reduce the numbers of polymorphonuclear leukocytes. After incubation the plates were rinsed and adherent cells (largely macrophages) were removed with rubber policemen, washed, viability was determined by dye exclusion (19), and cells were added in appropriate concentrations to Microtest II plate wells.

Studies to evaluate the effect of thymidine produced by PEC in these cultures were performed by separately maintaining B16 cells and PEC in the same concentrations and for the same interval as those used for measuring growth inhibition. Before harvest supernatant culture fluid was removed from B16 cultures and PEC supernatant culture fluid was then added to B16 cultures. These B16 cultures were pulsed and harvested as described previously. No differences in [^{3}H]TdR uptake between B16 cultures in medium and those in PEC supernatants were noted. These results indicated that inhibition of [^{3}H]TdR uptake in these assays was not due to competitive thymidine released from PEC.

Statistics. Statistical evaluation of survival experiments was made with the use of the logrank test as described (20).

Results. The effects of tilorone on B16 melanoma challenge of C57B1/6 mice were examined by injection of groups of mice (10 mice/group) with 10^4 B16 cells and ip treatment with 0.25 ml saline with or without 1 mg tilorone daily following tumor cell injection. Mice were observed for 90 days and tumor weights were obtained on the day of death. Survival data are shown in Fig. 1. Tilorone-treated mice showed lower incidence of tumor, increased survival time and lower average tumor weights than controls. Tumors were found in all of the control group and all mice in this group died. Forty percent of the tilorone-treated animals did not develop tumors during the period of observation. Tilorone-treated mice which did show tumor growth survived longer (45.5 $\pm$ 5.4 days compared with 34.4 $\pm$ 2.1 days) and had smaller tumors (5.6 $\pm$ 1.1 g compared with 9.8 $\pm$ 2.8 g). (Preceding data is shown as average $\pm$ SE.) Survival differences were significant ($P < 0.001$). Thus, tilorone protected C57B1/6 mice from challenge with the B16 melanoma.

The possible preferential toxicity of tilorone for B16 cells was examined in *in vitro* studies. Cultures of ^{51}Cr-labeled B16 cells or C57B1/6 fibroblasts were maintained in the presence of various concen-

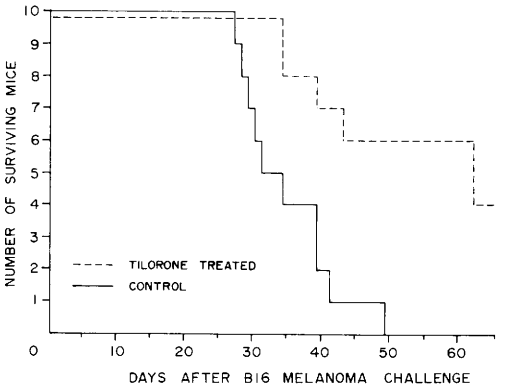

FIG. 1. Effect of tilorone on survival of C57B1/6 mice challenged with B16 melanoma. Mice were given 10^4 B16 cells sc and treated ip with 0.25 ml saline with (– – –) or without (——) 1 mg tilorone daily.

trations of tilorone. Supernatants were obtained at various intervals and compared with those from control cells maintained under similar conditions. Results are expressed as percentage release calculated from control values. As shown in Table I, the ability of tilorone to cause release of ^{51}Cr from labeled cells was measurable by 4 hr after the initiation of cultures in the presence of 100 μg/ml tilorone. Release was noted at 24 hr at the 10 μg/ml concentration. Tilorone in a concentration of 1 μg/ml did not appear to be toxic under these conditions. Results for B16 cells and for C57B1/6 fibroblasts were similar and indicated that tilorone did not have selective toxicity for B16 cells.

The effect of tilorone on cell growth was evaluated by culturing B16 cells or C57B1/6 fibroblasts in concentrations of tilorone ranging from 5.0 to 0.05 μg/ml. For this purpose 10^3 cells were added to wells of Microtest II plates and were maintained for 6 days with or without tilorone. Cultures received 1 μCi [^{3}H]TdR 24 hr before harvest. The results are shown in Table II and indicate that incubation of cells in 5 μg/ml tilorone resulted in almost complete inhibition of cell growth. B16 cell growth was reduced 53% and C57B1/6 fibroblast growth was inhibited by 91% at 1.6 μg/ml. No inhibition under these conditions was observed for either cell type at tilorone concentrations from 0.5 to 0.05 μg/ml.

Tilorone has been reported to enhance activity of the reticuloendothelial system (8) and to cause the release of cytokines from cells of this system (21, 22). In view of the recognized participation of macrophages in resistance to tumor growth the possibility that the protective effects of tilorone for B16 melanoma were partly mediated by enhanced macrophage activity was examined by means of *in vitro* cultures. PEC were harvested from control and tilorone-treated mice and added in appropriate concentrations to Microtest II plate wells. Test and control wells received 10^3

TABLE I. CYTOTOXIC EFFECTS OF TILORONE ON ^{51}Cr RELEASE FROM LABELED B16 CELLS AND C57B1/6 FIBROBLASTS

Tilorone (μg/ml)	B16[a]		C57B1/6 fibroblasts[a]	
	cpm ± SE	% release ± SE[b]	cpm ± SE	% release ± SE[b]
4 hr				
Control	725 ± 26	—	1274 ± 50	—
100 μg	1885 ± 15	65.3 ± 0.53	1579 ± 83	14.6 ± 3.5
10 μg	735 ± 12	<1	1282 ± 39	<1
1 μg	736 ± 26	<1	1240 ± 47	<1
24 hr				
Control	1150 ± 22	—	1839 ± 92	—
100 μg	2350 ± 81	83.3 ± 4.8	3096 ± 27	95.0 ± 1.7
10 μg	2155 ± 101	72.6 ± 6.2	2573 ± 147	55.0 ± 7.7
1 μg	1149 ± 14	<1	1820 ± 72	<1

[a] 10^4 labeled cells/ml culture. Cultures for each group were prepared in triplicate.
[b] Percentage release = (cpm of supernatant from cells incubated with test material) − (cpm of supernatant from control cells)/(cpm of supernatant obtained by lysis of control cells) × 100.

TABLE II. DOSE–RESPONSE EFFECTS OF TILORONE ON GROWTH OF B16 MELANOMA CELLS AND C57B1/6 FIBROBLASTS

	B16 melanoma[a]		C57B1/6 fibroblasts[a]	
	cpm ± SE	% growth inhibition[b]	cpm ± SE	% growth inhibition[b]
Tilorone (μg/ml)				
Control	5142 ± 202	—	2226 ± 69	—
5	97 ± 13	98	72 ± 2	97
1.6	2439 ± 248	53	219 ± 54	91
0.5	7443 ± 143	— (+44)	2478 ± 135	— (+11)
0.05	6115 ± 241	— (+18)	2253 ± 108	— (+1)

[a] 10^3 cells were cultured for 6 days with the indicated concentrations of tilorone. Cultures were pulsed 24 hr before harvest with 1 μCi[^{3}H]TdR per culture.

[b] Percentage growth inhibition = (cpm of control cultures) − (cpm of test cultures)/(cpm of control cultures) × 100.

B16 cells and plates were incubated for 6 days. Each well was pulsed with 1 μCi [^{3}H]TdR 24 hr before harvest. All groups were prepared in triplicate and the mean and standard error of the mean of [^{3}H]TdR uptake for each group were determined. The results of a representative experiment are shown in Table III. Minimal inhibition of B16 cell growth was observed at PEC:B16 ratios of 100:1. Lower ratios were not inhibitory. In contrast, tilorone-treated PEC demonstrated marked growth inhibitory effects. Virtually no uptake of [^{3}H]TdR was observed in cultures with ratios of 100:1 of PEC:B16 cells. Ratios of 10:1 inhibited growth by 58% and no inhibition was noted at 1:1. These results indicate a dose–response growth inhibition of tilorone-treated PEC for B16 cells.

To determine if the inhibition of B16 cell growth required viable PEC, appropriate concentrations of cells from either control or tilorone-treated mice were prepared, added to Microtest II plates and then frozen and thawed three times before the addition of 10^3 B16 cells to each well. The plates were then cultured for 6 days and 1 μCi [^{3}H]TdR was added to each well 24 hr before harvest. The results presented in Table IV show that lysates of tilorone-treated PEC exhibited a dose–response growth inhibition of B16 cells, whereas lysates of control cells stimulated growth. Similarly, tilorone-treated PEC and their lysates or supernatants inhibited growth of C57B1/6 fibroblasts (data not shown). Growth inhibition of B16 cells by tilorone-treated PEC was effected by the adherent cells and not by the nonadherent cells of the population. Thus, adherent PEC from tilorone-treated mice and products from such cells inhibit B16 cell growth in a dose-dependent manner.

TABLE III. GROWTH INHIBITION OF B16 MELANOMA CELLS BY PERITONEAL EXUDATE CELLS (PEC) FROM TILORONE-TREATED MICE

	Control PEC[a]		Tilorone-treated PEC[b]	
PEC:B16[c]	cpm ± SE	% growth inhibition[d]	cpm ± SE	% growth inhibition[d]
100:1	4615 ± 195	13	100	>99
10:1	6509 ± 129	— (+23)	2240 ± 674	58
1:1	6209 ± 220	— (+17)	5274 ± 324	<1

[a] PEC were obtained 5 days after ip injection of 2 ml thioglycollate.

[b] Thioglycollate injected mice were given 1 mg tilorone ip 24 hr before cell collection.

[c] 10^3 B16 cells were incubated 5 days, pulsed with 1 μCi [^{3}H]TdR 24 hr before harvest. B16 = 5308 ± 311.

[d] Percentage growth inhibition = (cpm of B16 control cultures) − (cpm of PEC:B16 cultures)/(cpm of B16 control cultures) × 100.

TABLE IV. GROWTH INHIBITION OF B16 MELANOMA CELLS BY LYSATES OF PERITONEAL EXUDATE CELLS (PEC) FROM TILORONE-TREATED MICE

	PEC lysate[a]			
	Control		Tilorone	
	cpm ± SE	% growth inhibition[b]	cpm ± SE	% growth inhibition[b]
PEC:B16				
100:1	8871 ± 315	— (+46)	100	99
10:1	8968 ± 564	— (+47)	4891 ± 221	20
1:1	7761 ± 15	— (+27)	6199 ± 70	— (+2)

[a] PEC were obtained 5 days after ip injection of 2 ml thioglycollate; 1 mg tilorone was given ip 24 hr before cell collection. PEC were added in appropriate concentrations to Microtest II plate wells. The plates were frozen and thawed three times before the addition of 10^3 B16 cells. Cultures were maintained 5 days and then 1 μCi [^{3}H]TdR was added to each well 24 hr before harvest. B16 = 6076 ± 104.

[b] Percentage growth inhibition = (cpm of B16 control cultures) − (cpm of PEC:B16 cultures)/(cpm of B16 control cultures) × 100.

Discussion. The results of this study have demonstrated that tilorone can decrease tumor incidence and prolong survival of mice challenged with B16 melanoma. These findings differ from those in which no protective effects of tilorone against B16 melanoma were observed (5, 10). The demonstration of protection here may have been due partly to the use of the minimum dose of tumor cells required to produce 90−100% tumor incidence in injected mice, thus permitting maximum opportunity for observation of alterations in tumor growth due to experimental manipulations. Although tilorone is toxic for B16 cells *in vitro*, this toxicity was not selective since C57B1/6 fibroblasts were comparably affected. The possibility that the inhibitory effects of tilorone on B16 melanoma growth was due not only to direct toxic effects for the tumor cells but also to effects on macrophage-mediated resistance to tumor growth was supported by observations that PEC from tilorone-treated mice, as well as lysates, and culture supernatants from such cells, inhibited growth of B16 cells *in vitro*.

The ability of tilorone to inhibit DNA polymerases (23, 24) probably accounts for the cytotoxic effects of this agent for various cells (4, 5). The growth inhibitory effects of tilorone for B16 melanoma noted in this study, in the absence of mouse toxicity, may have been a consequence of increased susceptibility of B16 cells *in vivo* because of the more rapid DNA synthesis exhibited by them than by host normal cells.

Tilorone has other biologic effects which may have contributed to its inhibition of tumor growth. For example, it is an inducer of interferon (1−3) which has antitumor activity (25), presumably because of its effects on the immune system (26). Further, tilorone enhances phagocytosis (8) and the release of cytokines (21, 22) by cells of the reticuloendothelial system (RES). Only the adherent cells from PEC preparations inhibited B16 cell growth *in vitro* supporting the idea that antitumor effects of tilorone are partly mediated through macrophages. Also, lysates or supernatants of PEC from tilorone-treated mice inhibited B16 cell growth in culture suggesting that macrophages may take up tilorone and release a toxic material which may be native tilorone, a toxic metabolite or other macrophage product. This possibility is supported by preliminary observations using [^{14}C]tilorone to evaluate uptake of this material by PEC obtained by thioglycollate stimulation as previously described. One milligram per mouse of labeled tilorone was injected ip 24 hr before cell harvest. Various concentrations of extensively washed PEC and known concentrations of labeled tilorone were counted. Results indicated that label associated with PEC was not readily removed by cell washing and that 10^5 cells could take up 0.3−0.5 μg of tilorone under these conditions. The observed toxicity of tilorone-treated PEC toward B16 cells may therefore have been partly due to tilorone taken up by PEC.

However, tilorone alone in a concentration of 1.6 μg/ml (0.32 μg/culture) inhibited growth of 10^3 B16 cells by only 53% (Table II), whereas 10^5 tilorone-treated PEC completely inhibited growth of the same number of B16 cells (Table III). The effective concentration of tilorone or its metabolites at the site(s) of tumor cell growth may have been greater than the amount indicated by binding studies due to the mobility and accumulation of macrophages and may have augmented in this manner the toxicity of macrophages, with bound tilorone, or their products for B16 cells. Thus, the inhibition of B16 melanoma growth by tilorone noted in this study could have been due both to primary effects, such as cytotoxicity mediated by inhibition of DNA polymerases, and to secondary effects, such as interferon induction and alterations of RES functions.

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