The Influence of Warfarin or Levamisole on the Incidence of Metastases Following Local Irradiation of a Solid Tumor

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KHT sarcomas were implanted in the right rear legs of C3H mice. An x-ray dose of 6000 rad, delivered in ten equal fractions over 12 days, resulted in 60% local tumor control, but 83% of these mice developed metastases. Three strategies to use the tumoricidal effect of x-radiation and reduce the incidence of metastases were compared. A modification of the fractionation scheme to deliver an initial large fraction of 1800 rad followed by seven 600-rad fractions resulted in a decreased incidence of metastases compared with the same dose delivered in ten equal fractions. The use of warfarin anticoagulation during the ten-fraction course of radiation resulted in a small decrease in the incidence of metastases. Immune stimulation with levamisole, injected subcutaneously every second day during the irradiation, also resulted in a decrease in the incidence of metastases. However, when warfarin or levamisole were combined with the eight-fraction radiation scheme there were fewer metastases than following the ten-fraction scheme. The combination of the eight-fraction radiation course with levamisole also produced a significant increase in primary tumor control. In this treatment regimen, therefore, levamisole appears to act as a radiation sensitizer. An hypothesis to explain this action is proposed.

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STUDIES, USING animal model systems have indicated that local irradiation of a solid tumor increases the incidence of metastatic disease.[1-4] Studies leading to a contrary conclusion have been less frequent.[5] The evaluation of clinical studies has led some investigators to suggest that radiation therapy, though effective as a modality for local tumor control, may be a factor in increasing the risk of distant metastases.[6-9] Other reports of clinical experience have concluded that irradiation has no effect,[10] or may even reduce the risk of distant metastases.[11]

Investigators have proposed a variety of mechanisms by which x-irradiation might influence metastatic spread. These have been reviewed by Cole[12] and Weiss.[13] A combination of at least three processes might be implicated: 1) the interaction of frequent tumor cell detachment and inherent mobility that are common properties of malignant neoplasms together with the increased tissue permeability that is a universal response to x-irradiation; 2) the sequestering of disseminated tumor cells in microthrombi; 3) the supression of cellular immune mechanisms which may develop following irradiation. A model system has been described in which mice bearing a KHT sarcoma transplanted in the leg show a predictable incidence and organ site of metastases.[3] A fractionated course of x-radiation appears to increase the incidence of metastases compared with nontreated mice. Using this model, the present study was undertaken to compare the results of three therapeutic strategies intended to use the effectiveness of x-radiation to achieve local control and, at the same time, to reduce the incidence of metastases. The first strategy was to make the initial fraction of radiation large so as to inactivate clonogenic cells that might be disseminated during the course of radiation. The second strategy was to have the mice in a state of hypocoagulation during the course of the radiation. The third strategy was to stimulate the host immune system. This report describes the effects of these strategies on the response of the primary tumor and on the incidence and sites of metastases.

Materials and Methods

Animals

Female C3H/He mice (Jackson Laboratories, Bar Harbor, ME) were used. The mice were maintained four to six per cage and offered food and water *ad*

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TABLE 1. Series A—Comparison of Untreated and Drug-Treated Mice

Tumor site	No treatment	Warfarin	Levamisole
Number of mice per group	21	18	21
Metastases	14/21 67%	13/18 72%	20/21 95%
Survival time with metastases (days)*	32 (20-40)	37 (26-49)	39 (25-50)
Survival time without metastases (days)*	29 (24-36)	40 (32-50)	34†
Lung	14	10	11
Mediastinum and chest wall	2	6	5
Axilla	5	4	11
Lumbar node	1	0	0
Ovary	2	2	1
Kidney	0	1	1
Other sites	0	1	1

* Values are average and range.
† Single animal.

libitum. Animals were 75 to 100 days old at the start of the experiment.

Tumor

The tumor was the KHT sarcoma originally arising in the ear of an untreated mouse.[14] This tumor has been maintained by serial passage using tumor fragments in C3H/He mice in this laboratory since 1967. Tumor passages 320 to 340 were used in this study. In all animals, the tumor was implanted by trochar (an approximately 1-mm^3 tumor fragment) into the subcutaneous tissue of the lateral aspect of the right thigh. The tumor becomes palpable after five to seven days. Mice with tumors were randomly allocated to the various treatment groups on day 7. Tumors were measured daily in three dimensions: Length, l, thickness t, and width w, using a micrometer and the volume calculated as $V = \pi/6(l \cdot t \cdot w)$. Tumor growth rates and doubling times were also derived from these measurements. Only tumors with a volume less than 500 mm^3 on day 10, *i.e.*, the start of radiation treatment, were used. Preliminary studies indicated that when the tumor grew to less than 500 mm^3 by day 10, the incidence of metastases, after leg amputation on day 10, varied from 25 to 50%.

Irradiation

Radiation was delivered using a 250 KvP x-ray machine filtered to an HVL of 1.4 mm^3. The dose of 6000 rad in ten equal fractions over 12 days was selected since preliminary data indicated that this treatment regime would result in a 50-60% local tumor control. The eight-fraction radiation course was to determine whether an initial large fraction of 1800 rads followed by seven 600-rad fractions would reduce the risk of metastatic spread compared with the same dose in rad but given in ten equal fractions. For irradiation, the mice were anesthetized with phenobarbitol, 35 mg/kg, ip, and placed under a lead shield so that only the tumor-bearing leg was irradiated.[15] For all irradiated groups, radiation started on day 10 after tumor implant.

Drug Treatments

The levamisole (Janssen R and D Incorporated, New Brunswick, New Jersey) was given by subcutaneous injection at a dose of 5 mg per kilogram of body weight every second day starting on day 9 after implant and ending on day 21.[16] Levamisole was always administered in the afternoon. The radiation treatments were always given in the morning thus no tumors were irradiated when levamisole could have been present at concentrations that might have exerted a radiation-dose-modifying influence on the tumor. The warfarin, 3-(acetonylbenzyl)-4 hydroxycoumarin, (Coumadin, Endo Laboratory, Garden City, New York) was administered in the drinking water at a concentration of 7.6 mg per liter. This concentration of warfarin has been found to be effective in maintaining prothrombin at 2 to 4 times normal levels.[17] Both levamisole and warfarin were started on day 9 after tumor implant and terminated on day 22 so as to be present during the irradiation treatments.

Three mice receiving warfarin only, and ten mice in the warfarin and irradiation groups died during the 13 days of drug administration. The mice were all anemic and had one or more sites where there was gross hemorrhage into the tissues. These mice that did not survive the treatment were excluded from the study. The toxicity of combined warfarin and levamisole at the concentrations used in this study made such a treatment combination impractical.

Criteria of Response

All mice were followed until death or until they were without tumor for 100 days. All mice were autopsied at death. Organs were examined grossly for evidence of tumor metastases, and suspicious lesions were fixed in formalin and examined microscopically. Metastases were considered present when tumor was found. In addition to the incidence and distribution of metastases, the following observations were made: 1) incidence of tumor control; 2) tumor growth rate; 3) survival time. The percent survival of mice with tumor(s) was plotted on a probit scale in order to transform the survival curves into straight lines. This demonstrated the differences in deaths due to the growth of the primary tumor (initial steep slope) and the late deaths that re-

sulted from metastatic disease. No data based on mice surviving less than three days after completion of the treatment *i.e.,* 25 days after implant, were used.

Treatment groups: There were three radiation treatment series each with three different drug treatment groups. Series A was to determine the incidence of metastases in the absence of radiation. Group 1 received no treatment, Groups 2 and 3 received either warfarin or levamisole.

Series B showed the effect of warfarin and levamisole on the incidence of metastases when the tumor was irradiated with 6000 rad delivered in ten equal fractions over 12 days. Group 1 showed the incidence of metastases with radiation only. Groups 2 and 3 showed the effects of warfarin or levamisole when combined with a ten-fraction radiation dose.

Series C was to determine whether a modification of the uniform fraction dose schedule using a large dose or radiation delivered at the start of the radiation treatment would inactivate enough clonogenic cells to reduce the incidence of metastases. Group 1 showed the effect of the radiation alone. Groups 2 and 3 showed the effect of warfarin or levamisole when combined with the eight-fraction radiation dose.

Results

Series A

By 10 days after implanting a tumor fragment, the KHT sarcomas were in exponential growth at rates similar to what others have reported for this tumor model.[14] With no treatment, the tumors grew to an average volume of between 5 and 6 cm^3 killing the mice by approximately 30 days after implant. The incidence of metastases in these mice was 67% with lung and right inguinal node always among the involved sites (Table 1). There was no statistically significant difference between the incidences of metastases in the nontreated mice (67%) and the mice treated with warfarin (72%). The higher incidence of metastases in the levamisole-treated groups, 95%, was statistically significant ($P < 0.05$) if compared with the pooled incidences of the nontreated and warfarin-treated groups. The lung was less frequently involved in the drug-treated mice than in the nontreated mice. This difference was not statistically significant. Warfarin and levamisole reduced the tumor growth rates compared with tumors in nontreated mice (Fig. 1). This resulted in a decreased mortality rate as shown in Figure 2. All animals showed some body weight loss during treatment. Weight loss was severe, 20 to 30% for mice receiving warfarin. These mice hemorrhaged easily, appeared to be anemic and often required their food to be softened before they could eat it.

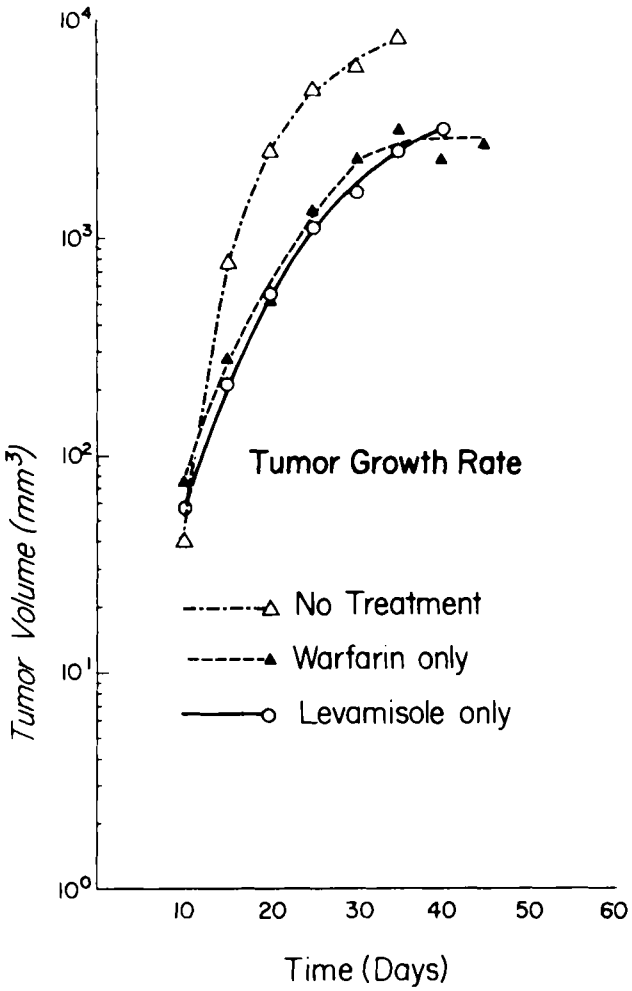

FIG. 1. Shows the tumor growth rates when the mice received no treatment, warfarin, or levamisole.

Series B

Following 10 fractions (6000 rad) of x-rays (Table 2), there was complete regression for 20 of 22 primary tumors. Two tumors were persistent. Of the 20 that regressed, eight recurred by 45 ± 12 days (mean ± SD). Of the remaining 12, ten mice died with metastases by 39 ± 4 days without a primary tumor and two mice survived over 100 days with no tumors, *i.e.,* total control.

Levamisole did not significantly ($P > 0.05$) change the proportion of tumors with complete regression or metastases compared with those exposed to irradiation only.

There was a decreased mortality rate (Fig. 3) for the mice treated by radiation and levamisole as shown by the slope of the survival curve. The displacement of the curve toward longer survival time shows there was an effect of levamisole on the radiation-induced growth inhibition of the primary tumor.

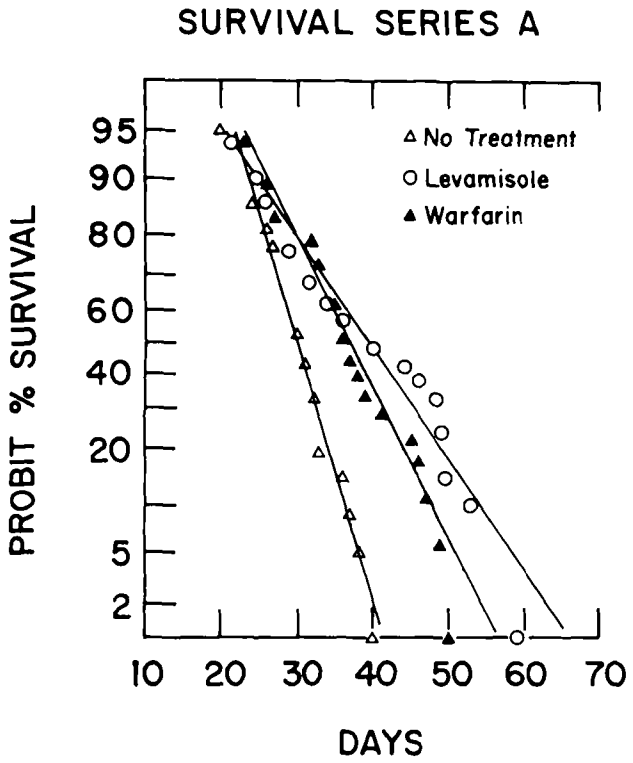

FIG. 2. Shows the survival of nontreated, warfarin- and levamisole-treated mice, Series A.

Series C

Following the eight-fraction irradiation regime (Table 2), there were fewer tumors (5/17) that were locally controlled than following the ten-fraction regime, which controlled 12 of 22. The incidence of metastases was reduced, 24% compared with 86% for the ten-fraction regimen, but the distribution of anatomic sites was similar. Compared with irradiation alone, warfarin treatment increased the proportion of complete regressions and primary tumor control. This, together with a decrease in the incidence of metastases, resulted in a 31% total tumor control.

Irradiation combined with levamisole resulted in complete regression of all the primary tumors. There were 13 of 18 that did not recur and seven of these had metastases. This resulted in 39% total tumor control. This was significantly greater ($P < 0.05$) than resulted from the eight- or ten-fraction irradiation-only treatments. The survival curves (Fig. 4) show that the combination of the eight-fraction radiation course with levamisole resulted in the survival curve being displaced toward longer survival times than any of the other treatment combinations. The mortality rate, as indicated by the slope of the curve, was similar to that observed for radiation only. The effect of levamisole was therefore to potentiate the radiation response of the primary tumor.

There were no statistically significant differences between the incidence of total tumor control following the ten- and eight-fraction irradiation-only treatments.

The irradiation regime using an initial large fraction did result in a decrease in the incidence of metastases. When this fractionation scheme was combined with warfarin or levamisole treatment there were fewer metastases (though not statistically significant) than when equal-sized fractions were given. Levamisole appeared to be especially effective both in increasing the incidence of local tumor control and in reducing the incidence of metastases.

Discussion

The data from this study concern three strategies intended to reduce the incidence of metastases when a solid tumor was treated by a fractionated course of x-radiation. Tumor cells lack the degree of adhesiveness that characterizes most normal tissues, and also possess an inherent motility.[12,13,18-20] These characteristics, to-

TABLE 2. Series B and C—Comparison of Radiation Schemes

	Series B—Ten-fraction radiation (6000 rad)			Series C—Eight-fraction radiation (6000 rad)		
Tumor site	X-ray	Warfarin	Levamisole	X-ray	Warfarin	Levamisole
Number of mice per group	22	17	18	17	16	18
Complete regression	20/22 91%	4/17 24%	16/18 89%	9/17 53%	12/16 75%	18/18 100%
Recurrence	8/20 40%	1/4 25%	3/16 19%	4/9 44%	4/12 33%	5/18 28%
Local control	12/22 55%	3/17 18%	13/18 72%	5/17 29%	8/16 50%	13/18 72%
Metastases	19/22 86%	13/17 76%	13/18 72%	4/17 24%	11/16 69%	11/18 61%
Total tumor control	2/22 9%	3/17 18%	5/18 28%	1/17 6%	5/16 31%	7/18 39%
Survival time with metastases	38 (30-62)	51 (36-106)	62 (45-90)	52 (29-110)	50 (26-90)	63 (47-112)
Survival time without metastases (days)*	61†	—‡	—‡	35†	—‡	—‡

* Values are average and range.
† Single animal.
‡ In drug-treated groups all mice without metastases were alive at 100 days with no tumor.

gether with the increased permeability of the vasculoconnective tissues induced by x-radiation could[21,22] facilitate the dissemination of potentially clonogenic tumor cells. One of the mechanisms that is believed to favor the establishment of metastatic foci from such cells is the inclusion of these cells in microthrombi, thus producing a sanctuary from the host cellular defense mechanisms and an environment that favors tumor growth.[13,18] Anticoagulation has been reported to reduce the incidence of metastases in some spontaneously metastasizing tumors.[23,24] Sodium warfarin is an effective anticoagulant and also inhibits tumor cell motility.[20,25] Warfarin alone did reduce tumor growth rate compared with tumors in untreated mice. It did not, however, change the incidence of metastases.

Following the ten-fraction radiation and warfarin regimen, only 24% of the tumors regressed compared with 91% in the mice receiving radiation only. This, we believe, is because the hypoxic component known to be present in this tumor[9,26] was further potentiated by the anemic condition of the mice, and the presence of hemorrhagic and necrotic foci in the tumors. Compared with radiation alone, warfarin treatment did not significantly reduce the incidence of metastases. Rotenger *et al.*[17] reviewed several clinical trials in which anticoagulation significantly improved the prognosis of patients receiving radiation therapy. In their laboratory study[17] using three different animal tumor models, single and fractionated courses of local radiation to the tumor failed to demonstrate any difference in tumor response between radiation alone and radiation combined with warfarin. Our data indicate that x-radiation combined with anticoagulation by warfarin results in a small enhancement (not statistically significant) of total tumor control. It seems quite possible that another fractionation scheme might be necessary in order to achieve the optimum advantage from combined anticoagulation and radiation therapy.

Some investigators have concluded that local x-radiation will inhibit the activity of the host cell-mediated immune defense mechanisms[27-29] and contribute to the increased risk of metastases.[30,31] Immune stimulation might, therefore, be expected to reduce the incidence of metastases. In the present study levamisole alone did not reduce the incidence of metastases and, in fact, this treatment group had a higher incidence of metastases than the nontreated or warfarin-treated groups. In mice where the tumors were locally irradiated, however, the levamisole reduced the incidence of metastases, increased the incidence of complete tumor regression, and decreased the incidence of local recurrences. The result was an increase in total tumor control. In this respect, levamisole seems to act as a radiosensitizer, especially for the radiation regimen using the large initial fraction.

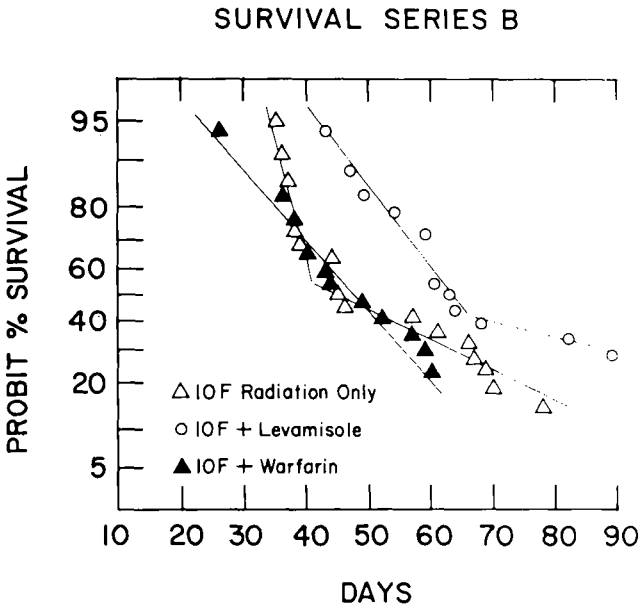

FIG. 3. Shows the survival of mice from Series B where the tumors were exposed to an x-ray dose of 6000 rad delivered in ten equal fractions over 12 days. The influence of warfarin and levamisole on survival are compared for this radiation schedule.

Levamisole is not known to be toxic to tumor cells.[32] Stewart *et al.*[33] found no potentiating effect of levamisole when combined with the local irradiation of a KHT tumor with single x-ray doses of 3000 rad. The same x-ray dose combined with levamisole produced a significant regression in the EMT-6 and 6C3HED tumor systems, both of which were more immunogenic than

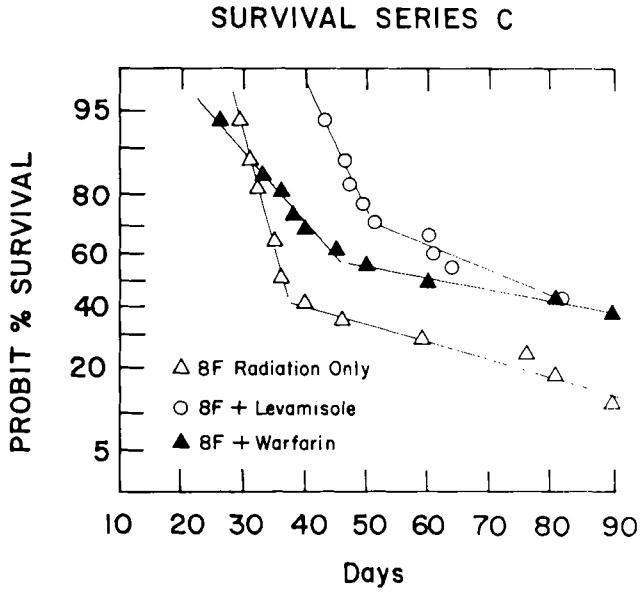

FIG. 4. Shows the survival of mice from Series C where the 6000-rad radiation dose was delivered in an initial large fraction of 1800 rad followed by seven equal fractions of 600 rad.

the KHT tumor. The differences in the effects of levamisole observed between our study and that of Stewart *et al*[33] may be related to the differences in radiation dose since the degree of immune stimulation was comparable.[32]

For those mice treated with radiation and levamisole, the initial portion of the survival curves (Figs. 3 and 4) were not different in slope compared with the mice receiving radiation only. This portion of the survival curve represented deaths from persistent and recurrent primary tumors. The levamisole treatment did, however, produce a displacement of this portion of the mortality curve toward longer survival. This delay in regrowth of the irradiated tumors could indicate fewer surviving clonogenic cells, suggesting that levamisole was acting as a potentiator of radiation injury. Levamisole has also been reported to extend the period of remission in clinical studies of combined therapies.[16,32] The second portion of the survival curve (Figs. 3 and 4) is due to death from metastatic tumors. The slopes of this portion of the mortality curves do not appear to differ between treatment groups, indicating similar growth rates for the metastases. While the combination of radiation and levamisole or warfarin reduced the frequency of metastases, they did not significantly influence the growth of metastases that did occur. To interpret the sensitizing action of levamisole treatment on the locally irradiated tumor, we suggest the following explanation.

One of the well-documented responses of Levamisole and some other nonspecific immune stimulators is to increase the number and activity of macrophages.[32,34-36] Such cells are cytotoxic to tumor cells. At each radiation fraction a proportion of clonogenic cells will have suffered sublethal[37] or potentially lethal damage (PLD).[38-40] Such cells, remaining *in situ*, could repair the radiation damage and be recruited into the growth fraction[41,42] when the extracellular milieu became suitable. However, in the host that had been treated with levamisole, we suggest that the stimulated macrophages might be differentially cytotoxic to the damaged tumor cells. A basis for this hypothesis comes from the observations of Vorbrodt *et al*.[43] These investigators have shown that macrophages may be preferentially cytotoxic to irradiated tumor cells whose cell membranes have been damaged by the radiation. If the hypothesis were correct, this would deplete the number of cells that could be recruited into the growth fraction and thus have the same effect on cell survival as if the levamisole had sensitized the tumor cells to radiation damage.[44,45] If this were the mechanism for the sensitizing action of levamisole, the distribution of metastases and their rate of growth would be unaltered but the number of clonogenic cells dispersed from the primary tumor would be reduced, leading to a decrease in the number of metastatic sites. Our data are consistent with this expectation.

For the KHT tumor model system the eight-fraction radiation course produced no improvement in primary tumor control but a decreased incidence of metastases compared with the course using ten equal fractions. When combined with warfarin or levamisole, however, the eight-fraction scheme increased the incidence of local control and decreased the incidence of metastatic disease. The large initial fraction would be expected to inactivate the oxygenated cells and largely deplete the growth fraction. The inactivation of clonogenic cells by the initial large dose followed by daily fractions would have also reduced the number of clonogenic cells available for dissemination, thus contributing to the reduced incidence of metastases. This same rationale, using a large initial x-ray fraction, has been proposed as the basis for several clinical radiotherapy trials.[46,47] The combination of levamisole and x-radiation therapy was an effective strategy both for improving local control and for reducing the incidence of metastases.

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