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Radiation-Induced Osteogenic Sarcoma of C3H Mouse: Effects of *Corynebacterium parvum* and WBI on its Natural History and Response to Irradiation*

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Abstract—*An osteogenic sarcoma, which appeared at* 316 days *following single dose* 5000 rad *to the leg of C3H mouse, has been studied as early generation F2 and F3 transplants in syngenic hosts with respect to local growth, pattern of spread, and response to local irradiation in normal,* C. parvum *treated, and whole body irradiated hosts. Mean survival of untreated mice was* 126 days *after transplantation. Grossly evident metastatic tumor appeared in* 85% *of these mice; in* 47 *of* 48 *animals metastases were seen in the lung.* Corynebacterium parvum *given intravenously as a single dose of* 350 μg *at* 96 hr *after tumor transplant retarded tumor growth: regression was observed in* 10 *of* 19 *tumors,* 3 *of* 19 *mice were cured of their osteosarcomas and mean survival was prolonged from* 126 *to* 173 days, *in one study where i.v.* C. parvum *was given when tumor was* 5 mm, 1 *of* 13 *mice was cured by* C. parvum *alone. Although* 5000 rad *resulted in* 100% *of tumor destruction in normal mice;* 49% *died of metastatic tumor to the lung. In* C. parvum *treatment mice only* 16% *died of metastatic tumor. The radiation doses which achieved control of half of the irradiated* 8 mm *diameter tumors were* 4350 *and* 3600 rad *for normal and* C. parvum *treated hosts, respectively. While body irradiation* 600 rad *given* 24 hr *prior to tumor transplant had an opposite effect to* Corynebacterium parvum.

INTRODUCTION

RECENT progress in the management of osteogenic sarcoma, by combination of intensive multidrug chemotherapy and surgery has been associated with improved survival figures: survival rate ≃ 20% at 5 yr following surgery alone [1] to 60–70% at 3 yr following chemotherapy and surgery [2, 3]. Of possible importance for further improvements are two findings: evidence of sarcoma associated antigens in some human osteosarcoma [4] and an apparent improvement in response rates to chemotherapy if given in combination with the non-specific immunopotentiator *C. parvum* [5]. For pre-clinical studies of new treatment strategies of human osteosarcomas we have investigated an osteogenic sarcoma which arose in one of our C3Hf/Sed mice. This paper presents results of these studies of the natural history and radiation response of this tumor in normal, immunopotentiated (*C. parvum* injected) and immunosuppressed hosts (whole body irradiation).

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*Supported in part by U.S. Public Health Service Grant CA 13311. The abbreviations used are: TCD_{50}, radiation dose which on the average would be expected to achieve control of one-half of the treated tumors; WBI, whole body irradiation; OGS–MGH, osteogenic sarcoma—Massachusetts General Hospital.

MATERIALS AND METHODS

Male and female C3Hf/Sed mice 10–12 weeks of age, from our defined-flora colony (bacterial flora were: *Bacillus* sp., *Clostridium* species and *Staphlococcus epidermides*) were used [6]. The osteogenic sarcoma studied in this experiment appeared at 316 days following 5000 rad (single dose) to the right leg of a 100 day old C3Hf/Sed mouse.

Histopathology of this tumor was reviewed by Dr. Louis Fagundes (Fig. 1). It is a highly cellular sarcoma characterized by pleomorphic spindle shaped tumor cells, abundant osteoid tissue and numerous blood vessels. On histological study, there is $\simeq 1$ mitotic figure per high power field. Nuclei are large with coarse chromatin. Cytoplasmic vacuoles are also seen occasionally.

For tumor transplantation, the second (F2) and third generation (F3) tumor tissue was harvested with aseptic technique and 1 mm^3 fragments were transplanted subcutaneously into the right flank or leg with a 13 gauge trocar. Three dimensions of the tumor were measured 3 times per week for the first 2 months, and then once a week until death of the mouse or end of the experiment. All mice were checked daily; post-mortem examinations have been performed to determine patterns of metastatic spread.

Radiation facility

For local irradiation, a specially designed small field irradiator was employed featuring parallel opposed 3 cm diameter ^{137}Cs portals [7]. Dose rate was 906 rad/min. The dose at 1 cm from the edge of the treatment field was 0.3%. Whole body irradiation was given in Gamma Cell 40 AECL ^{137}Cs at 117 rad/min to mice housed individually in a lucite carosel.

C. Parvum

C. parvum was provided as formalin-killed organisms suspended in thiomersalate Burroughs Wellcome Company, Reseach Triangle Park, N.C. through Dr. J. Whisnant. The material was sterile as determined by bacteriological studies in our laboratory. For injection, the preparation was diluted in 0.85% sodium chloride solution to obtain the desired concentration. *C. parvum* was given intravenously at 350 μg in 0.4 ml.

Experimental design

Three series of experiments have been performed. The second (F2) and third (F3) generations of isotransplants were used for the first two and third experiments respectively. The first consisted of studies of growth and pattern of tumor spread in normal, *C. parvum* treated and whole body irradiated hosts (WBI) using the second generation isotransplants (F2). Sixty mice were randomized into Control, WBI and *C. parvum* groups (20 mice per group). WBI mice received 600 rad single dose to the whole body at 24 hr prior to tumor transplants. *C. parvum* mice were given *C. parvum* 350 μg intravenously at 96 hours after the tumor transplantation.

The second series of the experiments were radiation dose–tumor control response assays with the second generation isotransplants (F2). One hundred and fifty-five mice were randomized into Control (55 mice), WBI (40 mice) and *C. parvum* (60 mice) (*C. parvum* 350 μg i.v. at 5 mm) groups respectively. Local irradiation of the tumor in the leg was performed on unanesthetized mice when the mean tumor diameter was 8 mm.

The third series of the experiments includes additional radiation dose tumor control response assay using a broader range of radiation doses and the third generation isotransplants (F3). Two hundred and fifty mice were randomized into 5 groups: Control, 70 mice; *C. parvum*, 80 mice, (*C. parvum* 350 μg i.v. at 5 mm tumor); WBI, 35 mice; (600 rad WBI 24 hr before transplant); *C. parvum* i.l. and i.v., 45 mice; (*C parvum* 350 μg intralesionally at 5 mm and intravenously at 8 mm), and WBI with C. parvum, 20 mice; (600 rad WBI 24 hr before transplant and *C. parvum* 350 μg i.v. at 5 mm and at 10 days after irradiation).

For radiation dose–tumor control response assays, single doses of radiation were given when the tumor reached 8 mm in diameter (250 mm^3) and results are expressed in terms of TCD_{50} or the radiation dose which on average would be expected to control half of the irradiated tumors.

RESULTS

Pattern of growth, spontaneous metastasis and survival of osteogenic sarcoma growing in the flank in control, C. parvum *and WBI treated mice*

The OGS–MGH (F2) reaches palpable size by 5–7 days after transplantation. With tumor growth, the tissues at the periphery become red and edematous but firm to hard at the core. Radiographs show heavy deposits of calcium consisted with the extensive osteoid seen microscopically (Figs. 1 and 2). Ulceration and bleeding are common for tumor diameter in excess of 20 mm. Growth curves for OGS–MGH in Control, WBI and *C. parvum* treated mice are shown in Fig. 3. In Control and WBI mice, volume doubling times over the volume range of 50–2000 mm^3, were $\simeq 11$ and 10 days respectively, with the WBI curve being displaced to the left $\simeq 6$–7 days. In contrast, the growth curve for OGS–MGH in

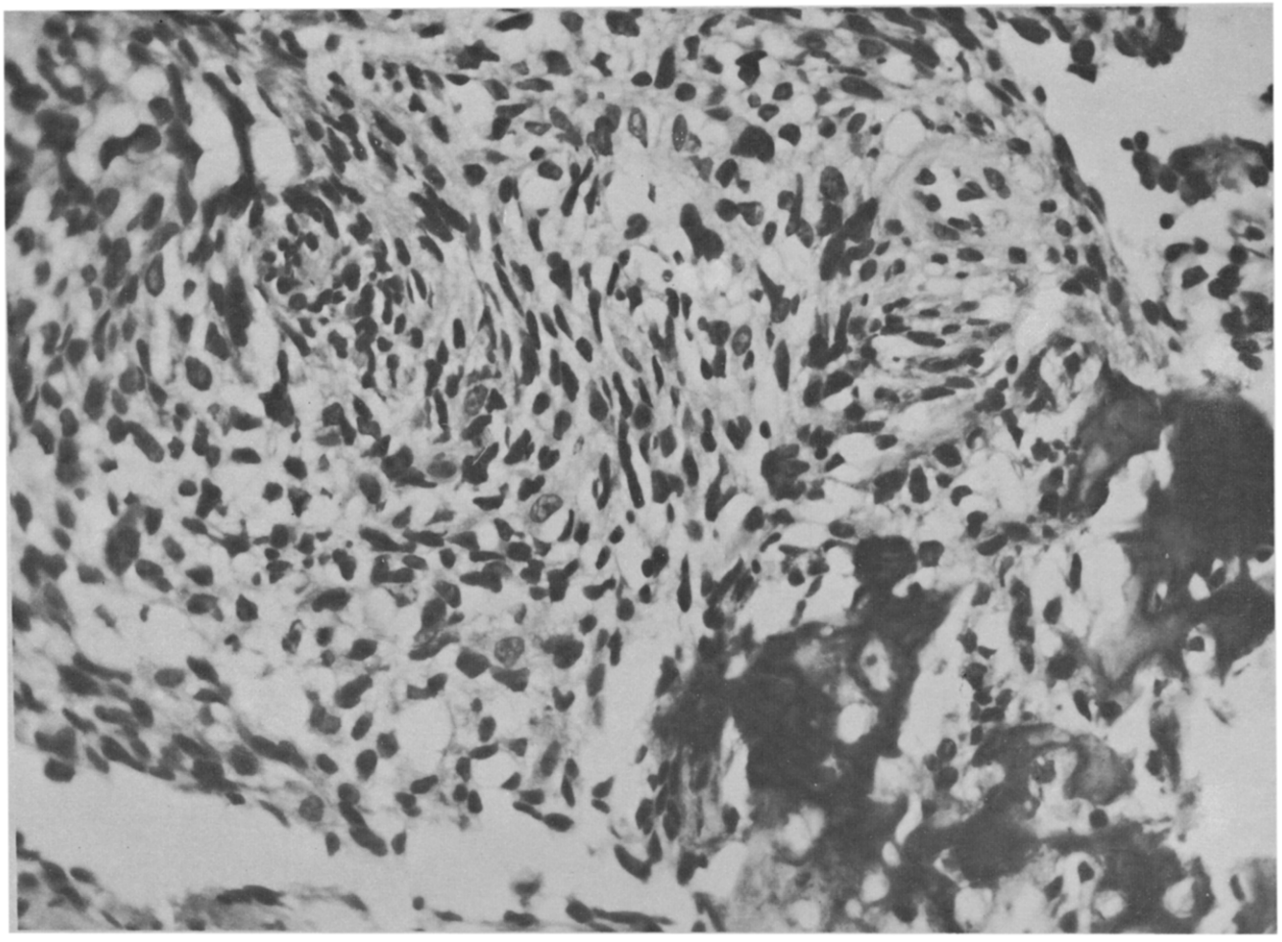

Fig. 1. *Photomicrograph of radiation induced osteogenic sarcoma. It is a highly cellular sarcoma characterized by pleomorphic spindle shaped tumor cells and numerous islands of osteoid* (*H* & *E* × 400).

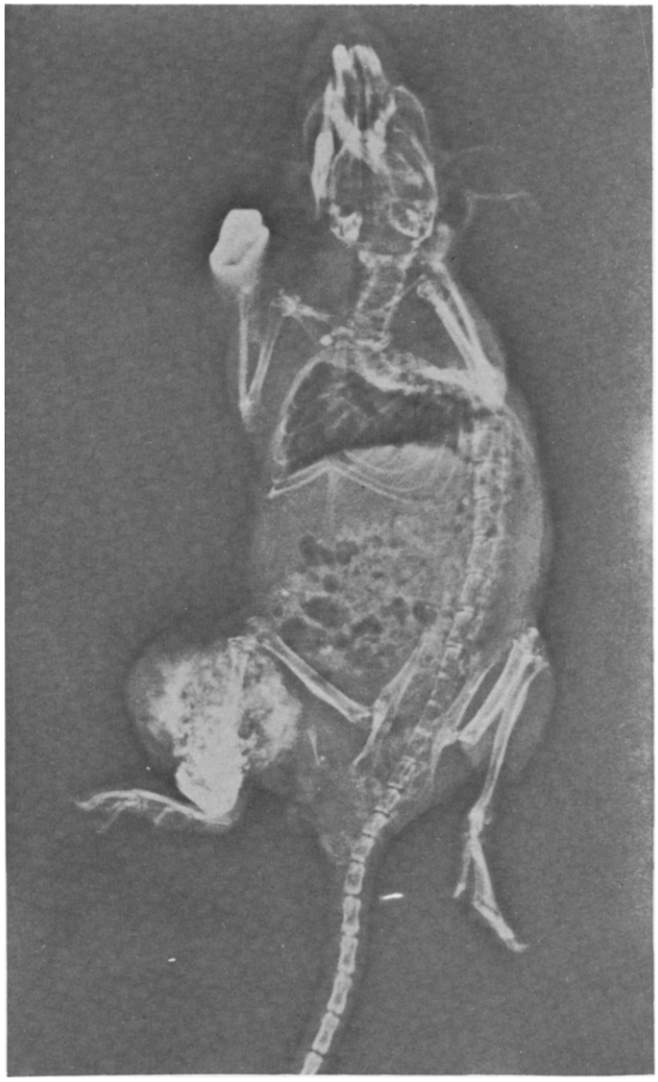

Fig. 2. Xerograph of a mouse with a transplanted osteogenic sarcoma growing in the right leg. The tumor mass and calcified osteoid are well outlined.

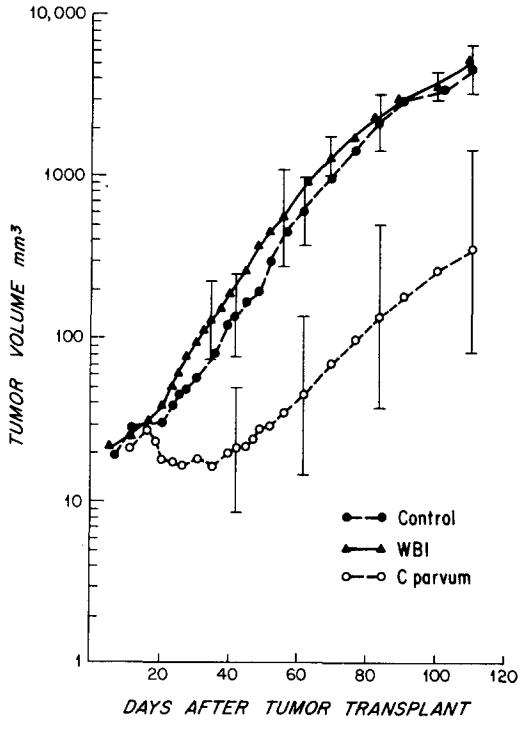

Fig. 3. Tumor growth curves of 3 groups expressed by mean tumor volume as a function of time in days. Vertical bars represent 95% confidence limits (Mean ± 1.96 S.E.). The difference of tumor growth curves between the Control and C. parvum *groups at* 42 days *and thereafter are statistically significant with* $P \leqq 0.001$ *(F and T test).*

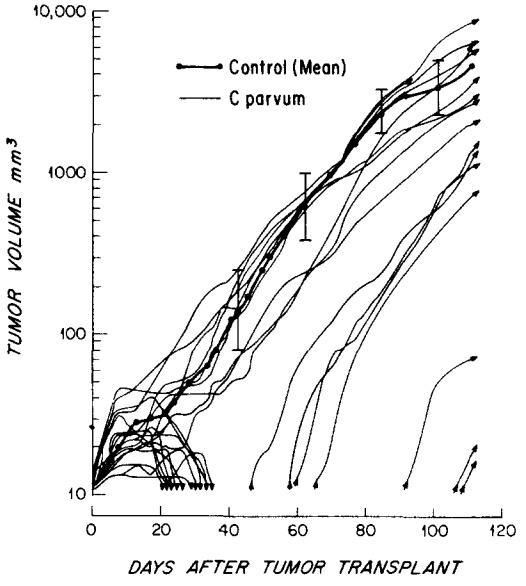

Fig. 4. Individual tumor growth curve of 19 *mice in* C. parvum *group is shown. For comparison, tumor growth curve of mean tumor volume of* 20 *tumors growing in Control group is also shown with vertical bars representing* 95% *confidence limits.*

the *C. parvum* mice, was displaced to the right of the control curve by ≃35–55 days and the volume doubling time was 15 days.

Growth curves of 19 individual tumors in mice which received *C. parvum* 350 μg i.v. at 96 hr after the tumor transplant are presented in Fig. 4. As a reference, the mean growth curve for 20 tumors in Control mice is also given. Ten of 19 tumors in the *C. parvum* group regressed to less than 10 mm^3 (3 × 3 × 3 mm). However, 7 of these 10 tumors recurred between 48 days and 116 days (median recurrence time = 70 days). Importantly, in 3 mice the regression was complete and permanent and those 3 mice are alive free of disease at 12 months after treatment. In no instance have we observed any indication of enhanced growth in the *C. parvum* treated mice.

Metastasis to the lung was a common occurrence in OGS–MGH mice; viz. incidences of pulmonary metastasis were 95%, 100% and 58% in Control, WBI, and *C. parvum* groups respectively.

Survival time was directly correlated with the frequency of spontaneous pulmonary metastases: mean survival times were 126, 115 and 173 days for the Control, WBI and *C. parvum* treated mice respectively.

Radiation dose–tumor control response of osteogenic sarcoma

Pooled data of the second (F2) and third (F3) experiments (395 mice) are shown in Table 1. Mice which died at ≦240 days after irradiation with distant metastases despite local control were excluded from the analysis of the dose–response relationship for local control. For all assays, there was a clear correlation between the frequency of local control and radiation dose. TCD_{50} values were 4350 rad (4000–4800)* and 3600 (3300–3900)* for this osteosarcoma in Control and *C. parvum* treated mice respectively. The TCD_{50} for OGS–MGH in *C. parvum* treated hosts is significantly less than in control hosts, $P < 0.01$. Administration of *C. parvum* 350 μg i.l. at 5 mm and i.v. at 8 mm tumor sizes was not more effective than the single i.v. dose at 5 mm tumor size. WBI 600 rad 24 hr before tumor transplant apparently had a small adverse effect. Regretably only a few mice survived free of metastases so that estimation of the TCD_{50} value for OGS–MGH in WBI mice was not possible.

*95% Confidence levels.

Table 1. *Local control of* 8 mm *osteogenic sarcoma* (*F*2 *and F*3) *growing in the leg and treated by local irradiation in normal hosts*

	Mice free of tumor/total treated at 240 days											
	Radiation dose in rad											
Assays	0	2100	2500	2750	3000	3250	3500	3750	4000	5000	6000	7000
Control		0/4	0/5	0/5	1/15	0/10	1/9	0/9	1/10	6/6	8/8	5/5
C. Parvum 350 μg i.v. at 5 mm tumor	1/13	1/5	0/4	0/10	4/17	1/10	7/10	5/10	5/9	6/6	7/7	8/8
C. Parvum 350 μg i.l. at 5 mm and i.v. at 8 mm	0/6		0/6		2/9		3/11					
WBI 600 rad 24 hr before tumor transplant			0/5		0/9		0/10		0/10	6/8	2/2	1/1

Pooled data of F2 and F3. TCD_{50} for the Control group is 4350 rad (95% confidence limit 3950–4750 rad) by logit method of analysis. *C. Parvum* 350 μg i.v. at 5 mm reduced TCD_{50} from 4350 rad to 3600 rad (95% confidence limit 3300–3900 rad).

Incidence of pulmonary metastases in mice with local control of OGS–MGH (F2) *by radiation* (>5000 rad)

As shown in Table 2, 49% (18/37) of Control mice which had control of the OGS–MGH in the leg following irradiation developed distant metastasis. The figure was 16% for mice treated with i.v. *C. parvum* at 5 mm but was 79% for mice subjected to 600 rad WBI at 24 hr before tumor transplant. In this experiment almost all deaths in mice which had local control of the leg tumor were due to metastasis in the lung.

Mean survival times of the mice which died of pulmonary metastases despite control of the tumor in the leg were 201, 222 and 157 days for Control, *C. parvum* and WBI groups respectively. The difference in survival times between Control and WBI groups was significant, $P<0.001$.

Table 2. Effects of C. Parvum *and WBI on pulmonary metastasis of mice with osteogenic sarcoma (F2) locally controlled by high dose local irradiation* (≧5000 rad)

	Pulmonary mets.
Control	18/37 (49%)
C. parvum 350 μg i.v. at 5 mm tumor	4/25 (16%)
WBI 600 rad 24 hr before tumor transplant	27/34 (79%)

The difference of frequency of pulmonary metastasis at 240 days between the Control and *C. parvum* group is statistically significant with $0.01 \leqq P \leqq 0.05$ by Chi square method. The increased frequency of pulmonary metastasis in WBI group is also statistically significant ($0.01 \leqq P \leqq 0.05$).

As shown in Table 3, the third generation (F3) of this tumor showed less frequent metastasis to the lung than the second generation tumor (F2). However, the numbers from the single experiment were too small to draw any meaningful conclusion.

Table 3. Frequency of pulmonary metastasis in the second (F2) and third (F3) generations of osteogenic sarcoma locally controlled by radiation

	Control	*C. parvum* 350 μg i.v. at 5 mm tumor	WBI 600 rad 24 hr before tumor transplant
F2	5/9	0/2	5/8
F3	1/4	0/14	1/3

The differences between F2 and F3 in Control and WBI groups are not statistically significant by Chi square method.

Tumor recurrence and survival time of mice with radiation dose <4000 rad *in control,* C. parvum *i.v.,* C. parvum *i.l. and i.v.* and *WBI group*

Tumor recurrence and survival times of mice which received radiation dose less than TCD_{50}, viz 4350 rad in the third series of the experiment (F3) were analyzed at doses ranging from 2500 to 4000 rad. As shown in Table 4, mean recurrence times were prolonged from 20 to 50 days (150%) by increasing radiation dose from 2500 to 4000 rad, $P=0.002$. Mean survival time was also improved by 42% (from 79 to 112 days), $P=0.003$. Administration of *C. parvum*, 350 μg i.v., at 5 mm prolonged tumor recurrence time from 29 to 52 days at 3000 rad, $P=0.03$ and from 50 to 70 days at 4000 rad, $P=0.039$. However, the improvement of mean survival time was not statistically significant. *C. parvum* 350 μg i.l. and i.v. had a similar beneficial effect to the mice as *C. parvum* 350 μg i.v. alone. WBI 600 rad 24 hr before tumor transplant showed no significant adverse effect in recurrence time and survival time over the control, see Fig. 5. The small component of

Pattern of tumor regression after high dose (5000–8000 rad) *local irradiation*

No regression of the tumor mass occurred even after high dose local irradiation (5000–7000 rad) which achieved permanent local control, see Fig. 5. The small component of

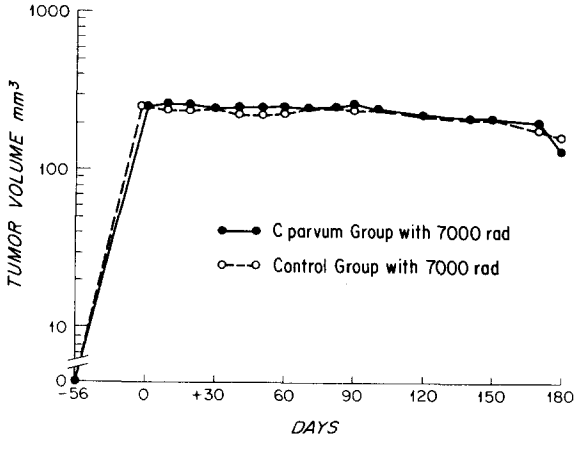

Fig. 5. Tumor regression curves of Control and C. parvum *groups after high dose local irradiation* (7000 rad). *There were* 10 *and* 8 *mice for Control and* C. parvum *groups at* 150 days. *No significant tumor regression was noted as a result of persisting calcified osteoid.*

Table 4. Mean tumor recurrence and survival times (days) *of mice with primary tumor* ($F3$) *unsuccessfully treated by radiation dose* $\leqq$4000 rad *in Control*, C. parvum *i.v.*, C. parvum *i.l. and i.v., and WBI groups*

	Radiation dose											
	2500 rad			3000 rad			3500 rad			4000 rad		
	Recurrence		Survival	Recurrence		Survival	Recurrence		Survival	Recurrence		Survival
Control	20	(5)	79	29	(14)	93	40	(8)	108	50	(9)	128
C. parvum i.v.	51	(4)	117	52	(13)	106	79	(3)	116	70	(4)	153
C. parvum i.l. and i.v.	25	(6)	87	81	(7)	104	79	(8)	151			
WBI	26	(5)	77	26	(9)	73	26	(10)	81	61	(10)	120

At radiation doses under TCD_{50} 4350 rad, mean tumor recurrence time was prolonged by *C. parvum* 350 μg i.v. at 5 mm tumor over the Control group at 3000 rad, 3500 rad and 4000 rad with $P \leqq 0.05$ (T test). In control group, mean survival and tumor recurrence times were also improved by increment of radiation dose from 2500 rad to 4000 rad, $P = 0.003$. There was also a strong trend of improved survival time by *C. parvum* i.v. at the dose levels studied over the Control group. Numbers of mice are given in parentheses.

soft tissue of the tumor regressed completely but the calcified core of the osteoid tissue persisted. When this bony core of the tumor became exposed, it was often eaten by the mice; these were the only instances of complete clearance. Histologic sections of residual palpable mass at 180 days after radiation doses $\geqq 5000$ rad (apparently controlled locally) did not contain tumor cells. An empty frame of calcified osteoid tissue and only a few scattered inflammatory cells were present.

DISCUSSION

Growth characteristics of the second generation isotransplants (F2) of OGS–MGH are: volume doubling time (VDT) of 11 days, a mean survival time (MST) of 126 days and distant metastases appearing in 85% of untreated hosts. These values may be compared with those for several osteosarcomas described in the literature: [8] osteosarcoma of C3He/6 mouse of Dunn, MST = 50 days, [9]; Ridgway osteosarcoma, MST = 25 days and VDT = 4–5 days [10]; Moloney sarcoma virus induced osteosarcoma of the Wistar–Lewis rat, MST = 19 days [8]; and a spontaneous canine osteosarcoma, VDT = 2–5 days [11]. These various tumor models also exhibited a very high incidence of distant metastases in the untreated host. In mice treated successfully for 8 mm OGS–MGH in the leg by local irradiation the incidence of distant metastases was reduced from 85% in the untreated or control mouse to 45%. This finding is consistent with the report that early amputation of the limb bearing Dunn's osteosarcoma was associated with a marked decrease in frequency of distant metastasis [9].

This tumor is relatively easily controlled by local irradiation viz a TCD_{50} for single dose irradiation of 4350 rad for 8 mm diameter lesions and $>90\%$ control rate at 5000 rad. In comparison, the TCD_{50} values for the non-immunogenic mammary carcinoma (MCa-MDAH-4) and the moderately immunogenic fibrosarcoma, FSa, (methyl cholanthrene induced) studied in this laboratory were 6500 and 3500 rad respectively [12]. These results contrast sharply with those for the Ridgway osteogenic sarcoma for which the TCD_{80} has been reported to be 4000 rad given in 5 treatments over 5 days [13].

Immunogenicity of experimentally induced osteogenic sarcoma has been described in terms of difference in TD_{50} for normal hosts and hosts which were immunized by implantation of radiation killed tumor fragments or by excision of subcutaneously transplanted tumors [14], and per cent of tumor cells killed *in vitro* by lymphocytes or serum of tumor bearing rats [8]. Osteosarcoma induced by chemical carcinogen (Cupric-chelated, *N*-hydroxy-2-acetylaminofluorene) was highly immunogenic [14]. FBJ virus induced sarcoma and Moloney sarcoma virus induced osteosarcoma in rats were consistently immunogenic [8, 14]. Weak immunogenicity or no immune response was detected in osteosarcomata induced by ^{90}Sr, ^{226}Ra and ^{32}P in mice and rats and spontaneous osteosarcoma have been described as weakly immunogenic or as non-immunogenic [14].

We studied immunogenicity of this tumor with non-specific immunopotentiator *C. parvum* and immunosuppressive agent WBI in terms of incidence of pulmonary metastasis, survival and TCD_{50}. *C. parvum* 350 μg i.v. at 5 mm caused a reduced incidence of metastases to lungs, prolongation of survival time, and a reduction of TCD_{50}. Extensive studies in our laboratory demonstrated that methyl cholanthrene induced fibrosarcoma was strongly immunogenic as shown by a 50% cure of mice bearing 5 mm tumors and reduction of TCD_{50} from 3400 rad to almost 0 rad by *C. parvum* 350 μg i.v. at 5 mm [12, 15–17]. A definite C. parvum effect has been described for two squamous cell carcinomas which had been induced by methyl cholanthrene [12] but virtually none for the non-immunogenic mammary carcinoma (MCa-MDAH-4) [12]. Immunogenicity of this osteogenic sarcoma induced by external irradiation is considered to be moderate to weak as other osteogenic sarcomas induced by internal irradiation with bone seeking radionuclides (^{90}Sr, ^{226}Ra, and ^{32}P) and spontaneous osteogenic sarcoma.

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