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Combination of Local Irradiation with Systemic Application of Anaerobic Corynebacteria in Therapy of a Murine Fibrosarcoma[1]

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SUMMARY

We studied whether local irradiation of a syngeneic fibrosarcoma in C3Hf/Bu mice could be made more effective by treating the tumor hosts with *Corynebacterium granulosum* or *Corynebacterium parvum*. Tumors were generated by transplanting fibrosarcoma cells into muscles of the right thighs or into the skin in the right flank of mice. When the tumors in the thighs grew to 8 mm in diameter, they were irradiated with 2500 or 3500 rads of γ-rays. *C. granulosum* (0.5 mg i.p.) was injected into the mice 3 to 4 days before irradiation or at 3 hr or 2, 7, or 14 days after irradiation. Irradiation alone with 2500 rads induced permanent regression of only 18% of tumors. *C. granulosum* increased curability to between 27 and 50%, depending upon the time of treatment with the bacterium. The treatment with *C. granulosum* did not change the effectiveness of 3500 rads: 80% cures compared with 82% in mice that received local irradiation. Doses of 0.5 mg and 0.25 mg *C. granulosum* given i.p. and i.v., respectively, and 0.25 mg of *C. parvum* given i.v. about 3 hr after local irradiation reduced the dose of irradiation yielding local tumor control in 50% of animals which was 3400 rads in control mice by about 1000 rads.

Fibrosarcomas growing in the skin were irradiated with 2200 rads when 6 mm in diameter. *C. granulosum* (0.25 mg i.v.) was given about 3 hr after irradiation. Irradiation induced 25% permanent cures, *C. granulosum* gave 77% permanent cures and the combination of the 2 cured all treated mice.

Animals in which tumors were not controlled locally were less likely to develop pulmonary metastases if they had received adjuvant immunotherapy; only 4% of mice with local recurrence after irradiation alone were free of pulmonary metastases compared with 19% of those animals treated with irradiation and anaerobic corynebacteria.

INTRODUCTION

Immune antitumor reaction is, in general, operative against a limited number of tumor cells; thus, it should be feasible to apply this reaction in the treatment of malignant tumors by combining it with conventional modes of cancer therapy. The efficacy of irradiation of tumors may depend not only on radiobiological factors (20) but also on the immunological competence of the host. Cohen and Cohen (3, 4) reported a significant increase in radiocurability of a murine mammary carcinoma by inoculating the tumor hosts with irradiation-attenuated fragments of the same tumor prior to local irradiation. In contrast, higher doses of irradiation were needed to cure this tumor when mice were immunologically suppressed by whole-body irradiation or corticosteroids (4). Powers *et al.* (11) observed an increase in TCD_{50}[2] values for the Gardner lymphosarcoma growing in immunosuppressed mice and a decrease in TCD_{50} values for the same tumors transplanted into mice that had been previously cured of this tumor. Suit and Kastelan (15) made similar observations for a murine methylcholanthrene-induced FSA. In addition, they observed a significant decrease in TCD_{50} value for FSA growing in mice specifically immunized with heavily irradiated tumor cells (15), but they were unable to demonstrate a benefit of stimulation of the immune system in the radiotherapy of a poorly antigenic mammary carcinoma (14). Haddow and Alexander (5) described an increase in response to irradiation of antigenic benzo(*a*)pyrene-induced rat sarcomas by inoculating the rats with autochthonous biopsied tumor pieces; systemic administration of *Bacillus Calmette-Guérin*, however, was ineffective. Recently, radiocurability of a murine FSA was augmented by treatment of tumor hosts with vitamin A (18). Also, treatment of mice with the methanol extraction residue fraction of *Bacillus Calmette-Guérin* (21) increased the radiocurability of a murine FSA and a mammary carcinoma. In this paper, we describe how treatment of mice with *C. granulosum* and *C. parvum* increases the efficiency of local γ-irradiation in controlling locally growing syngeneic FSA. These bacteria are known to be potent nonspecific immunostimulants (1, 12) that exhibit antitumor adjuvant activity (6, 7, 13).

MATERIALS AND METHODS

Mice. C3Hf/Bu mice of both sexes from our specific-pathogen-free breeding colony were used in this study. The mice carry only the following enteric bacteria: *Clostridium*

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[2] The abbreviations used are: TCD_{50}, dose of irradiation yielding local tumor control in 50% of animals; FSA, fibrosarcoma.

sp., *Peptostreptococcus* sp., *Bacillus* sp., and *Bacteriodes* sp. All mice used in each experiment were of the same sex and were 3 months old. The animals were kept 5 to a cage and maintained on a sterilized pellet diet and sterile water.

Tumor. FSA was induced in a young C3H/He female mouse by a single s.c. injection of 1 mg methylcholanthrene suspended in peanut oil (17). The 1st- through 4th-generation isotransplants of this tumor had been kept in a liquid nitrogen refrigerator, and the experiments were performed with tumors of the 5th generation. The tumor contains relatively strong tumor-specific antigen(s) (9, 15).

The suspension of single cells was prepared by digesting nonnecrotic tumor tissue with trypsin, a method described in detail previously (9). Viability of the cells was more than 95% as assessed by trypan blue exclusion and phase contrast microscopy. Tumors in the legs were generated by injecting 2×10^5 viable tumor cells into muscles of the right thighs of mice. Intracutaneous tumors were generated by injecting 7×10^5 viable cells into the skin of the right flank. The tumor cells were injected in a volume of 0.01 to 0.02 ml of Medium 199 (Difco Laboratories, Inc., Detroit, Mich.) supplemented with 5% of serum from normal syngeneic mice.

Local Irradiation. Tumors were irradiated when they reached an average diameter of 8 mm in the thigh and 6 mm in the flank skin. Mice were anesthetized with Nembutal sodium (0.07 mg/g body weight) (Abbott Laboratories, Chicago, Ill.) and taped on a brass plate. The leg or the skin bearing the tumor was then drawn away from the body and centered over a circular aperture 3 cm in diameter. The mouse on the plate was placed into the irradiator and rotated so that the aperture coincided exactly with an irradiation field 3 cm in diameter. The dose rate, measured with LiF thermoluminescent dosimeters, was about 1090 rads/min from 2 parallel opposed ^{137}Cs sources, each 17 cm from the tumor. Groups of mice received varying doses of irradiation to their tumors and were checked at least once per week for regrowth for up to 100 days. The end point of tumor response was local control by irradiation. In some experiments, the end-point used was the TCD_{50} (16).

Anaerobic Corynebacteria. Formalin-killed *C. granulosum* and *C. parvum* were obtained from the Pasteur Institute, Garches, France, and the Burroughs Wellcome Research Laboratories, Research Triangle Park, N. C., respectively. *C. granulosum* (Batch 5196) was supplied in 2-ml ampuls each with a bacterial concentration of 10 mg/ml. *C. parvum* (Batch PX 378) was supplied in 20-ml ampuls each with a bacterial concentration of 7 mg/ml. Doses of bacteria were 0.5 mg given i.p. or 0.25 mg given i.v. for *C. granulosum*, and 0.25 mg given i.v. for *C. parvum*. Bacteria were diluted with Solution A (8.0 g NaCl, 0.4 g KCl, 1.0 g glucose, and 0.35 g $NaHCO_3$ in 1 liter H_2O) so that each mouse received the desired dose in 0.4 ml.

Tumor Growth and Regression. To obtain tumor growth curves, 3 mutually orthogonal diameters of tumors were measured with vernier calipers, and the mean values were calculated.

RESULTS

FSA in Leg. Tumors 8 mm in diameter, growing in muscles of the hind legs of mice, were exposed to a single dose of 2500 or 3500 rads of γ-rays. *C. granulosum* (0.5 mg i.p.) was injected into the mice 3 to 4 days before irradiation or at 3 hr or 3, 7, or 14 days after irradiation. The results are shown in Table 1. Permanent regression was obtained in 18% of tumors receiving 2500 rads and 82% of those given 3500 rads. When irradiation with 2500 rads was combined with nonspecific immunostimulation, between 27 and 50% of animals were cured, depending upon the time of treatment with *C. granulosum*. In contrast, treatment with this bacterium did not influence the curative effect of 3500 rads. Tumors that did not regress in mice treated with both irradiation and *C. granulosum* grew more slowly than tumors treated with irradiation alone. *C. granulosum* alone slowed the growth of FSA 8 mm in diameter but caused no complete regression. The slowing of growth of tumors in mice that received combined therapy prolonged survival slightly. Thus, mice bearing tumors that did not regress following irradiation with 2500 rads lived 75.6 ± 4.4 days (range, 62 to 96 days) after irradiation as opposed to 84.3 ± 3.1 (range, 57 to 127 days) for those that received 2500 rads and *C. granulosum*.

To evaluate better the effectiveness of combined therapy, TCD_{50} assays were performed. Tumors were locally irradiated with doses ranging from 1400 to 4400 rads. Some mice received 0.5 mg of *C. granulosum* i.p. about 3 hr or at 7 days after irradiation or 0.25 mg of *C. granulosum* or *C. parvum* i.v. at about 3 hr after irradiation. Table 2 shows that treatment with *C. granulosum* or *C. parvum* reduced TCD_{50} values in controls from 3417 rads to between 2282 and 2777 rads. The reduction was slightly less if *C. granulosum* was given 7 days after irradiation. Both *C. granulosum* and *C. parvum* were more effective when combined with smaller doses of local irradiation, an observation similar to that made in the previous experiment (Table 1).

Treatment with *C. granulosum* reduced the incidence of pulmonary metastases in mice in which tumors did not regress. Forty-six mice that received irradiation only and died from regrowth of the local tumor were autopsied; only 2 were without metastases (4%). In contrast, 19% (18 of 95) mice were free of metastases after death from local regrowth of tumors treated by irradiation and anaerobic

Table 1

Control of FSA by local irradiation and injection of mice (tumor hosts) with C. granulosum

FSA in legs were irradiated when 8 mm in diameter.

Days *C. granulosum* (0.5 mg i.p.) was given before or after irradiation	Tumor control following irradiation with	
	2500 rads	3500 rads
−3 to −4	6/13[a] (46)[b]	11/12 (92)
0	3/11 (27)	7/10 (70)
2	3/9 (33)	8/9 (89)
7	5/10 (50)	8/9 (89)
14	3/10 (30)	6/10 (60)
	20/53 (38)	40/50 (80)
Control	2/11 (18)	9/11 (82)

[a] Number of mice cured over total number of mice.
[b] Numbers in parentheses, percentage of mice cured.

Table 2

Influence of treatment of mice with C. granulosum or C. parvum on irradiation response of FSA

FSA in legs were irradiated when 8 mm in diameter.

Irradiation dose (rads)	Control (irradiation) only	Irradiation + 0.5 mg i.p. of *C. granulosum* on Day 0	Irradiation + 0.5 mg i.p.. *C. granulosum* on Day 7	Irradiation + 0.25 mg *C. granulosum* i.v. on Day 0	Irradiation + 0.25 mg *C. parvum* i.v. on Day 0
1400			1/4 (25)	3/5 (60)	5/6 (83)
1900	1/9[a] (11)[b]	1/4 (25)	0/6 (0)	4/5 (80)	2/5 (40)
2200			1/6 (17)	3/5 (60)	3/5 (60)
2400	2/11 (18)	3/5 (60)			
2500			1/6 (17)	2/5 (40)	1/6 (17)
2700	3/11 (27)	2/5 (40)			
2800			3/6 (50)	3/5 (60)	1/5 (20)
3000	2/13 (15)	5/6 (83)			
3100			5/6 (83)	0/5 (0)	2/5 (40)
3300	7/12 (58)	4/5 (80)			
3400			4/6 (67)	1/5 (20)	4/5 (80)
3600	4/11 (36)	5/6 (83)			
3900	8/11 (73)	5/5 (100)	5/5 (100)	3/5 (60)	2/5 (40)
4400	5/5 (100)	5/5 (100)			
TCD^{50} values	3417	2431	2777	2373	2282
95% confidence limit	2995-3899	1940-3047	2374-3248	1416-3979	1095-4756

[a] Number of mice cured over total number of mice.

[b] Numbers in parentheses, percentage of mice cured.

corynebacteria ($p < 0.02$, χ^2 *test*).

FSA in Skin. An attempt was made to determine the effect of combined therapy on FSA growing in the skin. Mice with 6-mm tumors were either locally irradiated, treated with *C. granulosum*, locally irradiated and treated with *C. granulosum*, or left untreated. The dose of irradiation was 2200 rads of γ-rays and treatment with *C. granulosum* was 0.25 mg given i.v. approximately 3 hr after irradiation. Chart 1 shows that 25% of tumors (3 of 12) regressed when irradiated, 77% (10 of 13) in mice treated with *C. granulosum*, all tumors (10 of 10) in mice that received combination therapy and none (0 of 10) in those left untreated. Also, tumor regressions caused by the combined therapy occurred earlier than those caused by the single treatments. *C. granulosum* alone was more effective than irradiation, which was not the case with the tumors growing in the legs.

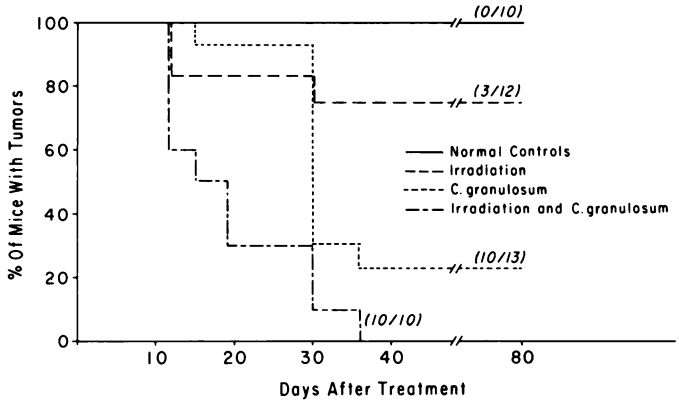

Chart 1. Percentage of C3Hf/Bu mice cured from FSA growing in the skin if treated with *C. granulosum* (0.25 mg i.v.), local irradiation with 2200 rads of γ-rays, or local irradiation plus *C. granulosum* given 3 hr after irradiation. Treatment was initiated when the tumors were 6 mm in diameter (Day 0). *Numbers in parentheses*, number of cured mice over total number of mice.

DISCUSSION

Our results demonstrate that the treatment of mice with *C. granulosum* or *C. parvum* bacteria can reduce the dose of γ-irradiation required to control a syngeneic murine FSA growing in the muscle of hind legs or in the skin. This was evidenced by a higher percentage of mice cured, and in mice not cured, by slower growth of tumors, prolonged survival, and reduced incidence of lung metastases. Both bacteria were equally effective.

One would anticipate that the antitumor immune response would be more pronounced in animals receiving higher doses of local irradiation to the tumor since it has to deal with fewer surviving tumor cells. However, both *C. granulosum* and *C. parvum* had a greater effect when combined with low irradiation doses. The reason for this is not established. It may be postulated that increased injury of stromal tissues (including blood vessels) at higher irradiation doses could interfere with the immune rejection response. However, Suit and Kastelan (15) showed that irradiation of the legs of mice did not significantly impair the local tumor rejection response, and we have shown that lower doses (up to 800 rads) do not affect the efficacy of nonspecific stimulation by *C. granulosum* in inhibiting artificial pulmonary metastases (8, 10). The release of more tumor antigens into the circulation at higher doses that may block immune lymphocytes (2) or cause immune paralysis (19) may also be a factor. Again, we have been unable to demonstrate any serum factors that interfere with the immune rejection response to this FSA (9), but rather we

have shown an increase in concomitant immunity to artificial pulmonary metastases when tumors in the leg are exposed to 6000 rads of γ-rays (unpublished observation).

Furthermore, at higher doses of irradiation the surviving tumor cells are hypoxic (because hypoxic cells are 2.5 to 3 times as radioresistant as oxic cells). The remoteness from the tumor vasculature of the hypoxic surviving tumor cells may protect them from immune mechanisms.

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