Brief Definitive Report

REGRESSION AND INHIBITION OF SARCOMA GROWTH BY INTERFERENCE WITH A RADIOSENSITIVE T-CELL POPULATION*

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Suppressor cells facilitate the growth of neoplasms that have tumor-specific transplantation antigens (TSTA) (1–7). In in vivo neutralization tests, suppressor cells, upon exposure to TSTA, enhance the growth of syngeneic sarcomas in both untreated and preimmunized hosts (7). These suppressor cells are recruited from a radiosensitive T-cell population which is present in the spleens of both unsensitized and tumor-bearing animals. In another system, the cells facilitating tumor growth have been shown to have surface markers that are characteristic of suppressor T cells and that are coded for by the I–J subregion of the H-2 complex (6). Despite the presence of suppressor activity, anti-tumor immunity can regularly be demonstrated both in vitro and in vivo in mice bearing small syngeneic tumors (8). Furthermore, this immunity, once generated, is fairly radioresistant (9).

Since radiosensitive suppressor cells facilitate tumor growth in vivo, since there is an anti-tumor immune response despite this suppression in tumor-bearers, and, since the immune response is fairly radioresistant, we hypothesized that whole body irradiation of mice with small tumors might preferentially interfere with the generation of suppressor cell activity, thus allowing a stronger anti-tumor immune response and consequent retarded tumor growth.

We show in this report that the growth of immunogenic tumors can be inhibited and even complete regressions achieved by whole body irradiation. Furthermore, we demonstrate that the essential effect of the irradiation is not on the tumor tissue itself, but most likely on host T cells. These observations suggest that in addition to the usual rationale for therapeutic uses of irradiation in cancer, treatment protocols that take into account an individual's suppressor mechanism may merit consideration in the future.

Materials and Methods

Experimental Outline. Mice were inoculated subcutaneously into both flanks with tumor cells, giving two potential tumor "sites" per mouse. The mice were randomized into various treatment

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groups (see Results); 6–8 days later, one group of mice was left untreated, and the other groups were given 400 rads of whole body irradiation. One group of irradiated mice was not further treated, whereas other groups were inoculated intravenously with spleen cells from nonsensitized, 6–8-wk-old BALB/c females. These spleen cells were either unfractionated, or they were enriched for, or deprived of T cells as described below.

The mice were examined twice weekly for tumor growth. Two perpendicular tumor diameters were measured. Mean tumor diameters in millimeters ($\bar{X} \pm SE$) for all sites per group were calculated, with negative sites being counted as 0. Statistical significance was estimated by Student's t test. All mice were ear-tagged. The treatment of the mice was unknown to the person scoring the animals.

Mice and Tumors. BALB/c mice were bred by brother/sister mating, and were regularly checked for their ability to accept intrastrain skin grafts. 6–8-wk-old females were used.

Two methylcholanthrene-induced sarcomas, 1315 and 1425 (10), were maintained by serial transplantation for 10–15 passages before use. Both possess strong individually unique TSTA, weak common tumor-associated antigens (10), and murine leukemia virus-associated antigens (11).

Tumor cell suspensions were prepared from fragments of healthy tissue by trypsinization (0.025% trypsin and 5 mM EDTA for 10 min), and they were washed in Waymouth's medium (Grand Island Biological Co., Grand Island, N. Y.). The mice were inoculated subcutaneously with 10^6 viable tumor cells into each flank.

Irradiation Procedures. Mice were given a sublethal dose (400 rads) of whole body irradiation from two opposing cobalt-60 sources.

Preparation of T-Cell-Enriched Spleen Cell Suspensions. Nylon wool columns to which non-T cells would bind preferentially were utilized according to Julius et al. (12). After column passage, 88–94% of the nonadherent cells were killed by a mouse anti-Thy-1.2 serum and complement, compared to 28–39% of the unfractionated spleen cells.

Preparation and Testing of a Goat Anti-T-Cell Serum. A goat was immunized three times subcutaneously with rat brain in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) to obtain antibodies cross-reacting to mouse T lymphocytes (13). The antiserum, which was heat-inactivated (56°C, 30 min) and exhaustively absorbed with BALB/c bone marrow cells, was tested on various BALB/c lymphoid cells for complement-dependent cytotoxicity, using a ^{51}Cr release assay. After the last absorption, bone marrow cells were not affected by the serum plus complement at all, whereas 90% of thymus cells and 25–35% of spleen cells were killed at a serum dilution of 1:50. Spleen cells incubated with 1:50 diluted antiserum and complement retained full ability to synthesize DNA upon exposure to lipopolysaccharide, while their ability to proliferate upon exposure to phytohemagglutinin was lost. This antiserum abolished the in vitro cytotoxic activity of alloimmune lymphocytes (14), as well as the in vivo reactivity of tumor-immune lymphocytes in (Winn) neutralization assays (unpublished observations).

To prepare T-deprived spleen cells for adoptive transfer, 2.5×10^7 cells/ml were suspended in Waymouth's medium, and an equal volume of 1:7 diluted goat anti-T serum was added. The mixture was incubated for 2 h at 4°C, after which pretested (nontoxic) rabbit complement (from 10-wk-old San Juan rabbits) was added to a final dilution of 1:20 and the mixtures were incubated at 37°C for 1 h. The cells were then washed twice, diluted in Waymouth's medium, and counted.

Results

Two preliminary tests showed that whole body irradiation of mice carrying sarcoma 1315 decreased tumor growth, provided that the irradiation was done 6–8 days after tumor transplantation (when the tumor just started appearing); no such effect of radiation was seen when it was given 10 or more days after transplantation. We decided, therefore, to start our treatments 6–8 days after tumor transplantation.

Three experiments were performed. In the first, 10^6 cells from sarcoma 1315 were transplanted subcutaneously into each flank of syngeneic BALB/c females. 8 days later, the mice were randomized into three groups of 10 mice each (Table I): group

TABLE I

Inhibition of 1315 Sarcoma Growth by Whole Body Irradiation (400 rads) of Mice Bearing Small Tumors

Group	Treatment of mice*		Tumor size at different time points after inoculation $\bar{X} \pm SE$ (20 sites/group)					Number of tumor sites with complete regression/total
			8 days	14 days	21 days	27 days	36 days	60 days
A	None		0.7 ± 0.2	4.1 ± 0.5	7.0 ± 0.9	11.3 ± 1.3	13.5 ± 1.4	0/20
B	400 rads		0.9 ± 0.1	2.5 ± 0.2	3.2 ± 0.7	3.6 ± 1.2	6.3 ± 2.1	7/20‡
C	400 rads followed by spleen cells, i.v.		0.9 ± 0.1	3.6 ± 0.4	5.6 ± 0.4	9.2 ± 0.5	>15.0	0/20
Statistical significance of differences between groups ($P <$)§		A-B	NS	0.01	0.01	0.001	0.02	0.004
		B-C	NS	0.05	0.01	0.001	0.01	0.004

* On day 1 the mice were inoculated subcutaneously in each flank with 10^6 sarcoma 1315 cells. Mice in groups B and C were irradiated on day 8, and mice in group C were also injected intravenously with 5×10^6 syngeneic spleen cells 2 h later.

‡ In all 7 cases, small tumors were observed to regress completely.

§ The statistical significance of the differences between groups in mean tumor diameter was determined by Student's *t* tests; significance in frequences of tumor regression was determined from Fischer Table. NS, not significant; $\bar{X}$, mean.

A was untreated, group B was given 400 rads of whole body irradiation, and group C was given 400 rads of whole body irradiation, followed in 2 h by the intravenous injection of 5×10^6 viable lymphoid cells from the spleens of normal BALB/c females. Tumor growth was significantly inhibited in the irradiated mice that received no spleen cells (group B). After an initial, weak inhibition seen in the irradiated group that received spleen cells (group C), tumor growth in this group was essentially the same as in the untreated mice (group A). Most remarkably, complete regression of tumors that had first grown to 2–4-mm diameters, was seen in 7 of 20 sites in group B; three mice had regression of their tumors on both sides, and remained tumor-free more than 3 mo later, by which time all the 20 control mice (groups A and C) and the remaining 7 mice in group B had died from tumor.

The lymphoid cells facilitating tumor growth upon adoptive transfer to irradiated mice were T cells. As shown in Table II, the growth of sarcoma 1315 was significantly inhibited by whole body irradiation (group B), and four complete regressions were observed. The therapeutic effect of the irradiation was counteracted by the intravenous injection of 2×10^7 unfractionated spleen cells (group C), thus confirming our observations in Table I. The tumors of the irradiated mice passively receiving T-cell-enriched spleen cells (group D) grew like the tumors in the untreated controls (group A), whereas the tumors of the irradiated mice receiving 2×10^7 T-depleted spleen cells (group E) grew much more slowly, and six of these regressed completely (and permanently).

In a third experiment, we also showed a therapeutic effect of irradiation on another sarcoma, 1425, which had been independently induced by methylcholanthrene. However, the effect of irradiation was less pronounced than with sarcoma 1315. The 1425 tumors in the irradiated mice receiving T-deprived spleen cells were significantly smaller after 15 ($P < 0.01$) and 20 days ($P < 0.001$) of growth than the tumors in the irradiated mice receiving T-enriched spleen cells, but this difference disappeared completely by 29 days of tumor growth.

Discussion

We have presented evidence that tumor growth can be inhibited significantly when

TABLE II

The Inhibition of Tumor Growth Caused by Whole Body Irradiation of Mice Carrying Small Tumors Can Be Prevented by Normal Syngeneic Spleen T Cells

Group	Treatment of mice*		Tumor size 6–41 days after inoculation $\bar{X} \pm SE$ (20 sites/group)				Number of tumor sites with complete regression/total
			15 days	20 days	29 days	41 days	
A	None		4.1 ± 0.3	6.2 ± 0.6	11.9 ± 0.7	13.6 ± 0.6	0/40
B	400 rads day 6		2.9 ± 0.2	2.8 ± 0.9	4.5 ± 1.5	7.5 ± 2.1	4/10‡
C	400 rads day 6 + 2 × 10^7 spleen cells, i.v.		2.8 ± 0.7	5.1 ± 1.3	10.6 ± 1.7	13.1 ± 1.0	0/10
D	400 rads day 8 + 2 × 10^7 T-enriched spleen cells, i.v.		3.9 ± 0.3	6.1 ± 0.5	10.2 ± 0.6	14.6 ± 0.3	0/20
E	400 rads day 8 + 2 × 10^7 T-deprived spleen cells, i.v.		2.9 ± 0.5	2.8 ± 0.8	5.6 ± 1.3	8.5 ± 1.5	6/20‡
Statistical significance of difference between groups ($P <$)§		A–B	0.05	0.01	0.001	0.001	0.002
		A–C	NS	NS	NS	NS	NS
		B–C	NS	0.02	0.02	0.05	0.05
		A–D	NS	NS	NS	NS	NS
		A–E	0.05	0.01	0.001	0.01	0.001
		D–E	0.005	0.01	0.01	0.001	0.01

* The mice were inoculated on day 1 subcutaneously in each flank with 10^6 cells from sarcoma 1315 (providing 2 tumor sites per mouse). 400 rads of whole body irradiation were given on day 6 or 8 after tumor transplantation. Spleen cells were inoculated intravenously 2 h after irradiation; procedures for obtaining T-enriched and T-deprived spleen cell suspensions are described in Materials and Methods.

‡ In all 10 cases, small tumors were observed to regress completely.

§ See footnote to Table I.

mice carrying small, subcutaneously transplanted tumors are given 400 rads of whole body irradiation. Most importantly, on several occasions complete tumor regressions were seen in irradiated mice carrying 1315 sarcomas. The cells affected by the radiation treatment were most likely lymphoid cells rather than tumor cells, since intravenous injection of spleen cells from nonsensitized, syngeneic mice abolished the radiation effect. The responsible spleen cells were T lymphocytes.

These observations support the hypothesis that a population of suppressor T cells exists in tumor-bearing animals where it plays an important role in facilitating the growth of immunogenic tumors. It also suggests that the suppressor cells, at least in the early phase of tumor growth (between 6 and 8 days), are recruited from a cell population which is sensitive to irradiation. At a later stage of tumor growth (10 days or later), a similar effect of whole body irradiation was not observed. This loss of the therapeutic effect of irradiation may be related to the eclipse phenomenon (15), and/or to the presence of appreciable amounts of circulating tumor antigens and other blocking factors (16) at that time, including those already formed by suppressor T cells (17). These phenomena are both related to tumor load (18). The development of resistant to irradiation could also be due to the presence of already activated suppressor cells which may have become less radiosensitive than the cell population from which they were derived (19). We are presently conducting experiments to explore these possibilities.

The therapeutic effect of irradiation was most dramatic against the 1315 sarcoma. Although this tumor, which we have studied extensively, does not regress normally, we observed about 30% permanent regressions as a result of irradiation in this study. Irradiation significantly retarded the growth of 1425 sarcomas, but this effect disappeared completely after 4 wk of tumor growth. The difference between these two tumors may relate to a difference in their immunogenicity, since 1315 is the more

immunogenic of the two in standard transplantation tests (10). Recently, we have obtained results similar to those for 1425 with another sarcoma, 1460, which is also less immunogenic than 1315. These observations emphasize the fact that each tumor is unique, and that different modalities may be required to achieve the same therapeutic effect.

We believe, nevertheless, that the approach described here, as well as the use of drugs such as cyclophosphamide instead of irradiation to preferentially interfere with suppressor cell activity while leaving the effector cell activities intact (20), can have general applicability to treating tumors of the mouse. An approach aimed at interference with suppressor cell activity may also have value as an adjunct to human cancer therapy.

We are anxious to point out, however, that we have not excluded that the radiation effect is due to the elimination of radiosensitive cells responsible for immunostimulation (21), and perhaps contributing to a stromal reaction contributing to the size of tumor nodules. This explanation is less likely, however, since our tumors grow well in nonimmunized BALB/c mice receiving 400 rads, and since permanent regression was seen in 17 of 50 tumor sites.

Summary

BALB/c mice were inoculated subcutaneously with 10^6 cells from either of two syngeneic sarcomas 1315 and 1425. 6–8 days later, the mice were randomized into groups which were left untreated or given 400 rads of whole body irradiation. Irradiation significantly retarded the growth of both sarcomas, and complete regressions were seen of ≅ 30% of the small, established 1315 tumors. The anti-tumor effect of irradiation was abolished if the irradiated mice were inoculated with a T-cell-enriched (but not with a T-cell-deprived) suspension of syngeneic spleen cells, suggesting that the irradiation inhibited tumor growth by affecting a radiosensitive population of host suppressor T cells.

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