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Treatment of the Mouse Lewis Tumor by the Association of Radiotherapy and Immunotherapy with *Bacillus Calmette-Guérin*[1]

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SUMMARY

Three groups of 6 to 8-week-old male mice with transplanted C57BL/6 × DBA/2 F_1 tumor were irradiated by a single dose of ^{60}Co localized on the tumor in association with i.p. injections of lyophilized *Bacillus Calmette-Guérin*. When the animals were sacrificed, tumor parameters (weight, surface area, and volume) were determined. Lung metastases were counted using a binocular magnifying glass. When *Bacillus Calmette-Guérin* injections were begun the day after the irradiation, tumor growth and the number of lung metastases were significantly decreased in comparison with the control group ($p < 0.01$). However, there was no significant difference between the control group, which received radiotherapy alone, and the groups in which *Bacillus Calmette-Guérin* treatment was started after the 4th day postirradiation. This emphasizes the importance of timing in combined radioimmunological treatment.

INTRODUCTION

The initial studies concerning the effects of adjuvants such as *Corynebacterium parvum* and BCG[3] on experimental tumor growth and the survival of animals were carried out in syngeneic and allogeneic ascitic tumors (3, 4). An identical type of study on solid tumors is much more recent, for example, the studies using BCG (2, 10, 20) or *C. parvum* in Betz sarcoma, Sarcoma 180, and carcinoma 755. Unfortunately, investigations involving immunological considerations such as the aforementioned studies and radiotherapy are actually not numerous. Despite certain experimental studies on the combined effects of irradiation and specific (17) or nonspecific (7, 18) immunotherapy on tumor growth, these new immunological methods are still the subject of much discussion namely, the use of immunotherapy in association with radiotherapy or chemotherapy in the recent results of treatment of acute leukemias (11, 13), malignant melanomas (6), and advanced cancers. Consequently, this study is set forth to evaluate experimentally the effects of radiotherapy alone and of the combination of radiotherapy and immunotherapy, using BCG, on local growth and distant metastases of a solid tumor *in vivo*.

MATERIALS AND METHODS

Male mice, C57 BL/6 × DBA/2 F_1, 6 to 8 weeks of age at the time of the experiments were used. Tumors were initiated in these mice by the implantation of Lewis tumor in the left hind paw. The Lewis tumor was transmitted *in vivo* from animal to animal of the same strain (5). The harvesting of tumor cells was carried out by dilaceration with wooden spatulas in a Petri dish that contained minimum essential medium and antibiotics. After filtration, viable cells were washed 3 times at 200 × *g*, counted using trypan blue, and then replaced in suspension to a concentration of 2×10^6 viable cells/0.20 ml of minimum essential medium. The cells were then injected into the muscles of the left thigh.

All animals received local irradiation to the tumor-bearing paw 8 days after implantation of the tumor, *i.e.*, 4 days after the appearance of the tumor. The area of the tumor was 120 ± 15 sq mm at the moment of irradiation. The irradiation was performed with ^{60}Co at a debit of 70 rads/min in air and a source-skin distance of 60 cm. The physical characteristics of the source were carefully verified before its utilization by use of ionization chambers (Baldwin type) and thermoluminescent dosimeters containing lithium fluoride. The irradiation was delivered strictly to the left paw using a fixed field and a single dose of 2100 rads. Radiation doses are specified as rads in the central portion of the tumor. The error in estimation of the dose is expected to have a standard error of 5%, including error from all other causes.

In addition to irradiation, all animals except the control were treated by i.p. lyophilized (Pasteur Institute) injections. Lyophilized BCG was chosen since encouraging results have been achieved consistently with humans (11, 13). Each injection delivered 1 mg of BCG contained in a 0.20-ml volume of 0.9% NaCl solution.

The evolution of the tumors in the mice was ascertained by evaluation of the primary tumor and its pulmonary metastases. Each week the tumor was measured with a slide caliper and the surface area was calculated by multiplying the 2 largest diameters. After the animal was sacrificed, the

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[3] The abbreviation used is: BCG, *Bacillus Calmette-Guérin*.

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tumor was weighed and the volume was determined by measuring height, width, and thickness with a slide caliper. The volume was calculated as for an ellipse using the formula $V = 4\pi abc/3 \times 6$. In addition, the pulmonary metastases were counted. These metastases appeared as round whitish nodules on the surface of the 2 lungs. The determination of metastases was made after the lungs were placed in a Bouin-Holland solution for 48 hr and counted on the surface of the pulmonary lobes with a binocular magnifying glass (×80).

The animals were distributed in the following groups. In Group 1, Subgroup A was composed of control tumor-bearing animals, *i.e.*, those that received irradiation but no BCG injections. Subgroup B received 3 injections of BCG before irradiation on the 2nd, 5th, and 8th days after tumor implantation, but no BCG following irradiation. In Subgroup C the injections of BCG were carried out 3 times, the day after the irradiation (D + 1) and then the 4th and 6th days after irradiation (D + 4 and D + 6). Subgroup D received BCG on Days 4 and 6 postirradiation. In Subgroup E, 1 injection of BCG was given on the 6th day postirradiation. Group 2 was similarly divided into 5 Subgroups in the following manner: *(a)* Subgroup A, controls; *(b)* Subgroup B, mice receiving BCG the day after irradiation (D + 1) and on Days 4, 6, 9, and 13 postirradiation; *(c)* Subgroup C, BCG injections on the 4th, 6th, 9th, and 13th days postirradiation; *(d)* Subgroup D, BCG injections on the 6th, 9th, and 13th days postirradiation; and *(e)* Subgroup E, BCG injections on the 9th and 13th days postirradiation. Group 3 was divided in the same manner: *(a)* Subgroup A, controls; *(b)* Subgroup B, mice receiving BCG the day after irradiation (D + 1) and on the 4th, 6th, 13th, 18th, and 26th days postirradiation; *(c)* Subgroup C, BCG injections on the 4th, 6th, 13th, 18th, and 26th days postirradiation; *(d)* Subgroup D, BCG injections on the 6th, 13th, 18th, and 26th days postirradiation; and *(e)* Subgroup E, BCG injections on the 13th, 18th, and 26th days postirradiation. The animals were sacrificed from Groups 1, 2, and 3, on the 15th, 23rd, and 30th days, respectively, after tumor implantation. For each of these 3 groups, there was a control Subgroup A′, which received no treatment.

All animals were randomly separated in different cages and lived in alternate 12-hr periods of light and darkness under constant conditions of temperature and ventilation.

All results were statistically interpreted by computer (Multi 20 Intertechnique) using the Student-Cochran test. Each group of values was evaluated for statistical significance ($p \leq 0.05$ was considered significant).

RESULTS

Group 1. The surveillance of primary tumor growth revealed differences in the subgroups of each group of animals. In Group 1, in which the tumor developed for the shortest period of time (15 days) and in which the number of injections of BCG was the smallest, the increase in tumor surface (Chart 1) was much less for Subgroups B and C ($p < 0.01$). There was no significant difference between Subgroups D and E and the control Subgroup A ($p > 0.2$).

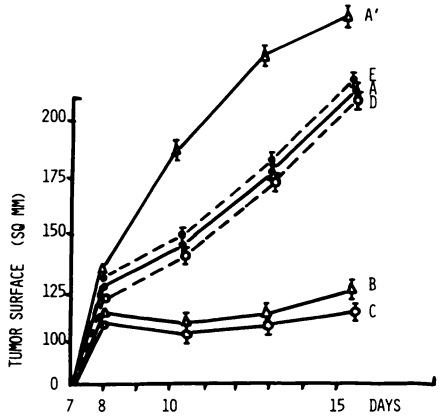

Chart 1. Tumor surface in Group 1. Demonstrates clearly the significant difference of tumor surface evolution between Subgroups B and C (corresponding respectively to the BCG inoculations before the irradiation and those started immediately after irradiation) and control Subgroup A. However, in Subgroups D and E, in which BCG is started after the 4th postirradiation day, the evolution of the tumor surface is comparable to that of Subgroup A, having been exposed to radiation and not to BCG. The aspect of this curve, which corresponds to Group 1 (tumor evolution of 15 days), is similar to that of Groups 2 and 3 (tumor evolution of 23 and 30 days, respectively).

The study of the weight and volume of tumor (Table 1) in Group 1 showed differences between the 5 subgroups comparable to the study of the evolution of tumor surface. The tumor weight was diminished in Subgroups B and C but only for Subgroup C was it statistically significant ($p < 0.05$). The same proportional differences were found in the average tumor volume in the 5 subgroups of Group 1. Counting of metastases in Group 1 (Table 1) revealed a net decrease in the number of metastases for Subgroups B and C as compared with the control Subgroup A ($p < 0.01$), and an increase not statistically significant ($p > 0.1$) for Subgroups D and E as compared with the controls.

Group 2. In Group 2, in which the tumor developed for 23 days, the determinations of tumor surface showed differences among the subgroups that were comparable to the differences noted in subgroups of Group 1. In effect, 15 days after irradiation, the tumor surface was less in Subgroups B and C than in the control Subgroup A ($p < 0.01$). At the same time the tumor surfaces for Subgroups D and E were greater than for control Subgroup A ($p < 0.01$). The study of the tumor weights and volumes (Table 1) showed the same modifications as those observed in Group 1; namely, a decrease in the weight and volume of tumors for Subgroups B and C ($p < 0.05$), and a significant increase of these parameters for Subgroups D and E as compared to control Subgroup A ($p > 0.3$). The number of metastases (Table 1) also decreased in Subgroups B and C in comparison to control Subgroup A ($p < 0.05$). In Subgroups D and E the number of metastases was not significantly different from that in control Subgroup A ($p > 0.3$).

Group 3. The observation of tumor evolution in Group 3 (30-day tumor evolution) showed results similar to those found in Groups 1 and 2, *i.e.*, a net decrease in tumor surface, weight, and volume (Table 1) for Subgroup C. Similarly, the number of pulmonary metastases (Table 1)

Table 1

Tumor volume and number of pulmonary metastases

Statistical interpretations of tumor volume in cu cm and of number of pulmonary metastases are considered at the moment of animal sacrifice for all groups and subgroups. As for tumor surface area and weight, the data show the interest of BCG administration with radiotherapy as indicated by the comparison of control Subgroup A (radiotherapy alone) and A′ (no treatment). These data are substantiated only if the BCG is begun up to and including the 4th postirradiation day. Beyond the 4th postirradiation day, there is no significant difference between Subgroups D and E (irradiation plus BCG) and control Subgroup A (radiotherapy alone).

	Tumor volume (cu cm)			No. of pulmonary metastases		
Subgroups	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
A	3.4 ± 0.9[a]	5.5 ± 1.1	6 ± 1.1	15.3 ± 0.9	85 ± 2	85 ± 1.5
A′	5.9 ± 0.9	8.83 ± 1.45	10.8 ± 2.2	35 ± 0.9	82 ± 1	78 ± 1.4
B	3 ± 0.3	3.3 ± 0.7	5.5 ± 0.8	12.5 ± 1.03	55 ± 1.5	75 ± 1.3
C	2 ± 0.4	3 ± 0.4	4.8 ± 0.8	5 ± 0.7	37 ± 1.5	45 ± 1.4
D	2.5 ± 0.5	7.03 ± 0.75	6.6 ± 0.9	17.5 ± 1.1	73.1 ± 0.8	75 ± 1.3
E	3.5 ± 1.01	7.1 ± 1.2	7.3 ± 1.3	17.6 ± 1.7	75 ± 0.7	85 ± 0.9

[a] Mean ± S.E.

was fewer in Subgroup C than in the control subgroup ($p < 0.05$).

DISCUSSION

Our results show a statistically significant decrease in tumor growth in animals treated with BCG before and after irradiation. In the latter this was true only if BCG therapy was begun immediately after irradiation. Growth of the primary tumor, as studied by surveillance of tumor surface and measurements of its volume and weight, as well as the appearance of pulmonary metastases, was significantly diminished for all sacrificed groups that had received BCG immediately after irradiation, irrespective of the duration of tumor evolution. The number of injections of BCG had no influence on tumor growth or on the number of pulmonary metastases. There are limitations in the experimental conditions, since the tumor was chosen for its rapid evolution. This is not perhaps the ideal experimental model for long-term immunotherapy, in which repeated injections of immunostimulants are given. However, it is the early initiation of BCG therapy that is important.

Effectively, there was no statistically significant difference between control Subgroup A and the subgroups in which BCG was started after the 4th day postirradiation. This was true for all 3 groups, regardless of the number of injections of BCG given and the duration of tumor evolution. These results were in agreement with the observations of a number of authors who reported that a greater number of injections of BCG did not always bring about a better result (19). Furthermore, the results delineate an increase in tumor growth for the subgroups in which BCG injections were initiated the latest. Although not statistically significant, this increase was observed in 3 groups, especially in Groups 2 and 3.

There was a good concordance and homogeneity in our results with the use of lyophilized BCG. Some authors have obtained concordant results with fresh BCG (Pasteur Institute) and sometimes discordant results with lyophilized BCG (9, 12). Our results are contradictory to the latter.

The effect of BCG is remarkable when one considers the rapid evolution of the experimental Lewis tumor. This tumor metastasizes very quickly to the lungs and is considered to be one of the most resistant tumors to all types of therapy.

Certain authors (8, 15, 18, 19) have stressed the importance of the moment of initiation of active nonspecific immunotherapy on tumor evolution. Milas *et al.* (14), during the study of fibrosarcoma growth in the C3Hf/Bu mouse demonstrated that the number of lung metastases was significantly reduced if the mice were treated 2 days after receiving tumor cells, but not if they were treated 7 days after tumor implantation.

The lapse of time between the implantation of the experimental tumor and the initiation of immunotherapy is of great importance. It seems that immunotherapy can be active only if the tumor mass is not too significant. Some investigators have indicated that the creation of experimental leukemias or solid tumors necessitates a greater number of cells when the animals have been previously immunostimulated with BCG (1, 11, 13). This condition is acquired either at the beginning of tumor evolution or immediately after a cytoreduction treatment such as radiotherapy.

This study shows that BCG is only active on the growth of the Lewis tumor or on the appearance of its pulmonary metastases when it is used before irradiation or immediately afterward. In consideration of our experimental results demonstrating the importance of sequential radioimmunotherapy, postradiotherapy immunodepression, as cited by certain authors (16), should be corrected by the use of immunostimulants at the termination of radiotherapy.

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