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Transfer of Age-associated Restrained Tumor Growth in Mice by Old-to-Young Bone Marrow Transplantation[1]

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ABSTRACT

B16 melanoma and Lewis lung carcinoma grow more slowly in aged mice. Immunesenescent changes may account for this age-related difference. To test for the effect of immune deficiency on the growth of these tumors, we treated young mice with an immunosuppressive dose of radiation and then observed tumor growth. We also radiated young mice to a higher (lethal) dose and then rescued them with either young or old bone marrow transfusion. Tumors grew more slowly in radiated mice than controls and in those reconstituted with old bone marrow. These findings support the concept of immunesenescent-related reduced tumor growth.

INTRODUCTION

In mammalian species when sexual maturation occurs, there begins a gradual involution of the immune system (11, 16), an occurrence which has been causally linked to increased neoplasia with aging (7). Although tumor prevalence is clearly greater in immune-deficient populations, tumor growth rate and metastatic potential may, in fact, be reduced. For example, we found that, when B16 melanoma cells are implanted s.c. in the flank of C57BL/6 mice, tumors grow at a slower rate and reach a final volume that is less when the recipient host is old (4). Furthermore, when the B16 cells were injected i.v. into young or old mice, the number of pulmonary melanoma colonies enumerated 3 weeks later was also less, and survival was greater for the older animals (4). Similar observations have been made on a murine alveolar cell carcinoma (18). In those studies, tumor growth was also slower in mice immunocompromised by radiation or corticosteroids but was enhanced when splenocytes from young healthy mice were transfused syngeneically to irradiated animals just prior to tumor inoculation. The occurrence of metastases, however, was greater in the immunosuppressed animals.

We speculated that the reduced rate of tumor growth and metastases observed in the B16 melanoma model was due, at least in part, to immune senescense. To prove this, we attempted to create a similar immune deficiency in young mice by either sublethal radiation or lethal radiation with infusion of old bone marrow and subsequently observed tumor growth rates. Such treatment resulted in decreased tumor growth rates but greater numbers of metastatic colonies after i.v. tumor cell inoculation.

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MATERIALS AND METHODS

Mice. Young (2 to 3 months) C57BL/6 mice were purchased from the Charles River Breeding Laboratories, Inc. (Wilmington, MA), and old mice were obtained through a pilot award from the National Institute on Aging. These mice were from special National Institute on Aging aging colonies maintained at the Charles River Breeding Laboratories and shipped to the Animal Care Facility at the University of Vermont at 23 months of age. They were maintained with regular photoperiods, isothermal conditions, and laboratory chow and water *ad libitum*. For each experiment, a minimum of 8 animals was allocated to each group.

Radiation and Bone Marrow Reconstitution. Young (2 to 3 months) mice were administered total body irradiation from a 1.2 MeV cobalt 60 (Theratron Junior; Atomic Energy of Canada, Ltd.) source. The mice were placed in a Lucite chamber and exposed at a dose rate of 43.8 rads/min to a total dose of 500 rads. Such treatment has been shown to result in depressed immune function (12, 18). One day after radiation, mice were subjected to experimental tumor inoculation (see below). To confirm that 500-rad treatment did immune suppress these mice, antibody response to tetanus toxoid primary and secondary immunization was compared in radiated and nonradiated mice by a protocol described previously (2). In additional experiments, young mice were radiated by a similar technique but to a total dose of 950 rads. One hr later, these mice were administered bone marrow prepared from either young (2 to 3 months) or old (24 to 25 months) syngeneic healthy donors. To prepare this marrow, the young or old donors were sacrificed by cervical dislocation, and each fermur was disarticulated and stripped of muscle. Small needle punctures were made at each end, and the marrow cavity was flushed with sterile 0.9% NaCl solution (saline). Bone marrow cells were washed in 0.1 M Dulbecco's phosphate-buffered saline, pH 7.2, adjusted to 10^7 nucleated cells/ml, and injected (0.1 ml) into the lateral tail vein of recipient mice. Mortality in the posttransplant period was generally between 25 and 50%, with the peak death rate at 10 days and no deaths occurring beyond 3 weeks posttransfusion. There was no difference in mortality between those given young bone marrow and those given old. Furthermore, by 3 weeks, surviving mice appeared healthy, and there was no weight difference between the experimental groups. At 1 month, posttransplant mice were inoculated with tumor cells as described below.

Experimental Tumors. Lewis lung carcinoma (3LL), an undifferentiated murine squamous cell carcinoma syngeneic to C57BL mice, is maintained *in vivo* in our laboratory by serial passage. For these experiments, cells were prepared for injection by processing freshly resected solid 3LL tumor with a tissue homogenizer (Dounce), and a single cell suspension was prepared. Viable cells, determined by trypan blue exclusion, were adjusted to 10^6 cells/ml, and 0.1 ml of cells was injected s.c. in the right flank of young (2 months) or old (24 months) mice. Palpable tumor was measured in 2 perpendicular axes with tissue calipers twice weekly, and tumor volume was estimated assuming spherical growth by the formula $\frac{4}{3}\pi r^3$.

B16 murine melanoma (F_1 and F_{10} sublines) was generously provided by the National Cancer Institute's Tumor Repository, Frederick, MD. The B16 cells were stored frozen, thawed, and grown in minimal essential medium supplemented with 10% (v/v) fetal calf serum and antibiotics. For each experiment, cell viability was ascertained by trypan blue exclusion, and the cells were diluted to 10^6 viable cells/ml. For all experiments, 10^5 cells/mouse were administered.

F_{10} cells were injected s.c. into the right flank, and animals were examined daily for survival and tumor growth. For these experiments, the F_{10} B16 melanoma line was chosen because preliminary studies revealed that rapid and reproducible local growth occurred after s.c. implantation. In additional experiments, F_1 cells were injected i.v. (lateral tail vein), and the mice were sacrificed 2 weeks later. Lungs and other organs were examined under a dissecting microscope for the presence of colonies of melanoma growth. The F_1 line of B16 was chosen for these experiments because our preliminary experiments had indicated that this tumor line given i.v. resulted in a reproducible small number of pulmonary colonies which could be accurately quantified.

Statistical Analysis. The difference between experimental groups in tumor growth, pulmonary colonies, or antibody production was analyzed for statistical significance by the Student *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

Growth s.c. of 3LL, Young *versus* Old. In our earlier experiments, an age advantage had been demonstrated in host response to B16 melanoma. To confirm that a similar advantage may be present in a different experimental tumor, 3LL was inoculated s.c. in young or old mice. Chart 1 demonstrates the observed reduced tumor growth in the old mice. Tumor volumes were significantly smaller at each measurement day in the older animals ($p < 0.05$). For example, at Day 15, the mean volume was 1783 ± 142 (S.E.) cu mm *versus* 158 ± 57 cu mm, and at Day 21, 4258 ± 445 cu mm *versus* 698 ± 151 cu mm for young and old mice, respectively. The mean survival time after tumor inoculation was greater for the old mice [32 ± 4 (S.E.) days *versus* 29 ± 5 days, statistically not significant], and the final tumor volume (last measurement prior to death) was less (3,303 ± 592 cu mm *versus* 11,589 ± 1,573 cu mm for old and young, respectively; $p < 0.05$). These findings were similar to those that we observed with B16.

Radiation-induced Immune Suppression. To test the hypothesis that immune senescence accounts for, or contributes to, the observed age advantage in these tumor models, we attempted, by radiation, to immune suppress young mice and observe tumor growth and metastases. Treatment with 500 R has been shown to suppress various immune functions (12, 18); however, to confirm that that dose had a similar effect in the strain of mice selected for these studies, antibody response to tetanus toxoid immunization was tested. Chart 2 depicts the results. There were 10 mice in each group, and the mean antibody level (absorbance) at each time point is shown. Mice pretreated with radiation had less antibody production after the primary immunization. Response to the secondary immunization 4 weeks after the radiation dose and primary immunization, however, was brisk.

Tumor Growth and Colonization in Irradiated (Immune Suppressed) Mice. In groups of mice that received 500 R, growth of B16 melanoma was slower (Chart 3), and mean survival was slightly longer (34 ± 7 days *versus* 30 ± 7 days; not of statistical significance). These findings were consistent with our hypothesis that (radiation-induced) immune deficiency would be associated with slower tumor growth; however, other radiation-induced factors or alterations were not excluded. Furthermore, the occurrence of pulmonary melanoma colonies 3 weeks after i.v. injection of B16 cells was greater for the animals having received radiation (Chart 4), a finding that is consistent with either immune deficiency (10) or radiation-induced pulmonary endothelial injury (15) or both. In earlier experiments, however, we have shown that pulmonary melanoma colonies were fewer after i.v. injection of B16 cells in aged mice (4). We would therefore favor the latter hypothesis (radiation-induced endothelial injury) as the mecha-

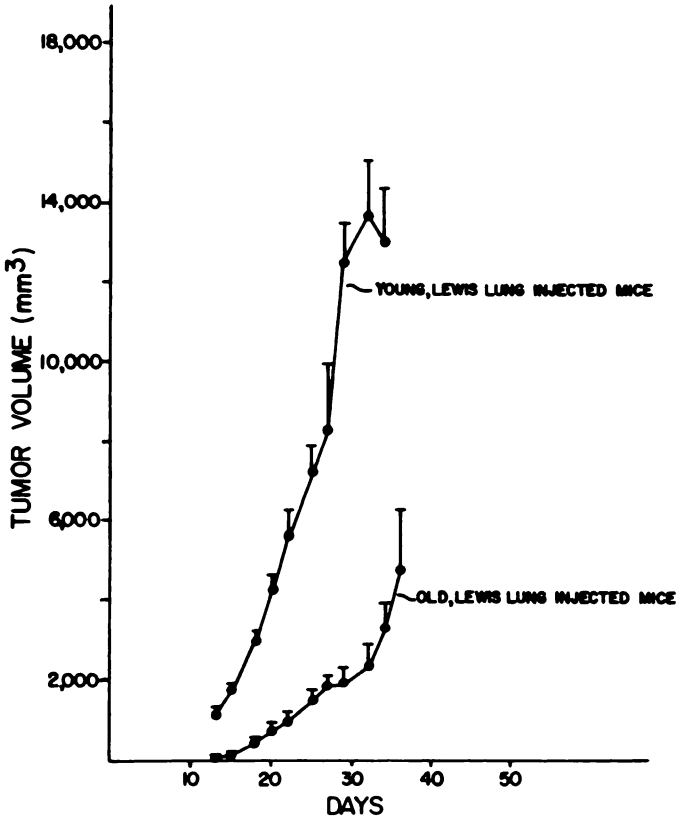

Chart 1. Tumor growth in young and old mice. Lewis lung carcinoma was injected s.c. in the right flank of young and old mice ($n = 10$ in each group), and tumor size in 2 dimensions was measured and tumor volume calculated. The rate of tumor growth and final tumor volume were greater in the young animals. *Bars*, S.E.

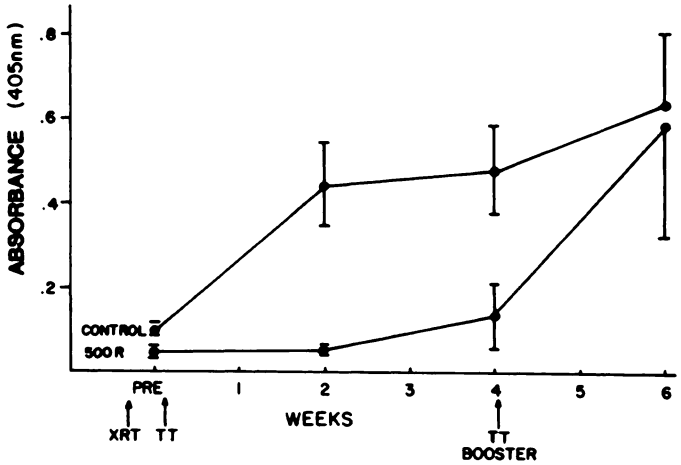

Chart 2. Antibody production in mice after sublethal radiation. Mice radiated with 500 R were subsequently inoculated with tetanus toxoid (*TT*), and antitetanus toxin was determined by an enzyme-linked immunosorbent assay. It is apparent that mice pretreated with X-ray therapy (*XRT*) had less antibody production after the primary immunization. Response to secondary "booster" immunization 4 weeks after the radiation was brisk. *Bars*, S.E.

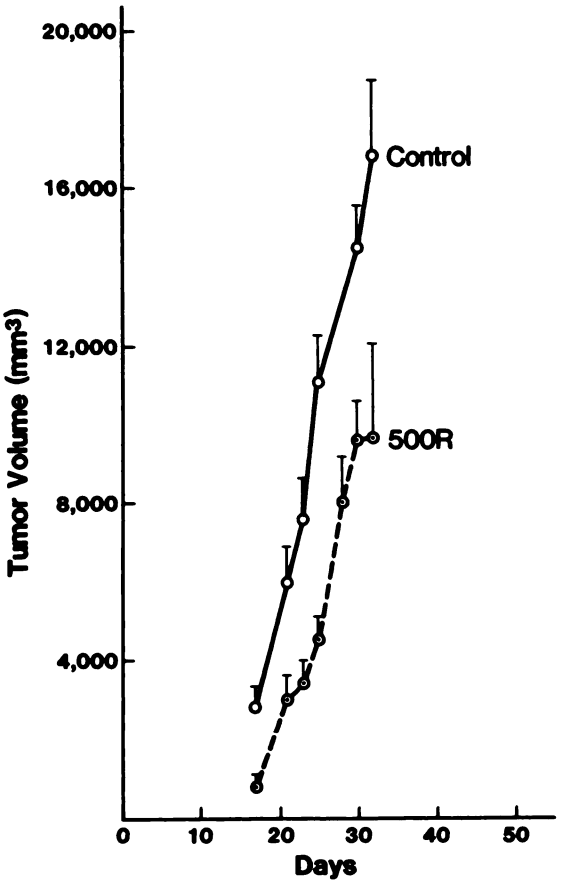

Chart 3. B16 melanoma growth after sublethal radiation (500 R). The F_{10} line of B16 melanoma was injected s.c. into young mice 24 hr after receiving the radiation. Tumor diameters were measured twice weekly, and volumes were calculated. For each group of animals, the mean tumor volume at each time point is depicted. By Day 23, and each day thereafter, the tumor volume was significantly less ($p < 0.05$) for the mice pretreated with radiation. *Bars*, S.E.

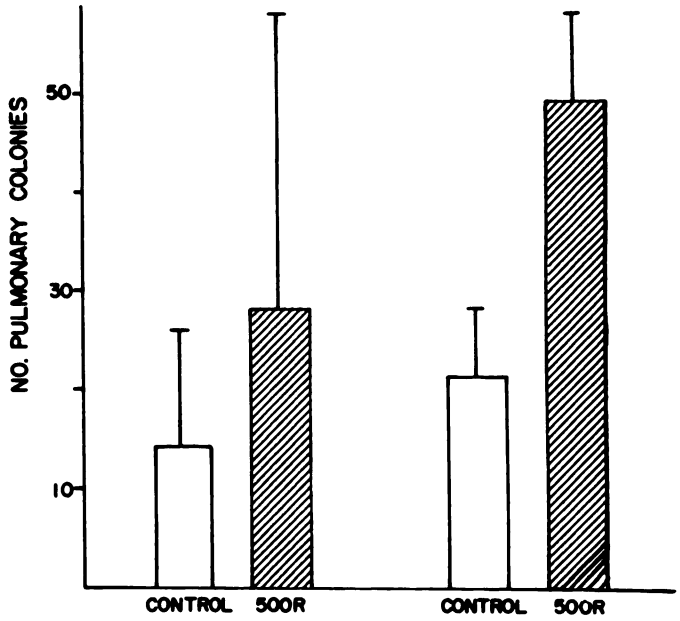

Chart 4. Pulmonary melanoma colonies in irradiated and control mice. Mice were injected with B16 melanoma cells by the lateral tail vein, and 3 weeks later, pulmonary colonies were enumerated. The mean number of colonies for each experimental group for 2 such experiments is depicted. A greater number of colonies was observed in the mice pretreated with sublethal radiation [Experiment 1 (*left*), not statistically significant; Experiment 2 (*right*), $p < 0.05$]. *Bars*, S.E.

nism of increased colony formation in these experiments.

Tumor Growth in Lethally Irradiated Young Mice Reconstituted with Either Young or Old Bone Marrow. In an effort to further evaluate the importance of immunological factors accounting for the observed age advantage in local tumor growth, we reconstituted young mice with either young or old bone marrow and later observed B16 growth rates. As can be seen in Chart 5, we found that tumor growth was most rapid for the nonradiated, nontransfused controls, intermediate for the irradiated mice reconstituted with young marrow, and the slowest for the irradiated mice reconstituted with old marrow. The difference in tumor volume for the latter groups was statistically significant at each of the observation dates.

DISCUSSION

We demonstrated reduced tumor growth rates in old mice, in mice treated with sublethal doses of radiation, and in mice treated with lethal doses of radiation followed immediately by reconstitution with bone marrow from old donors. The implication that immune deficiency (associated with aging or radiation) accounted for the growth retardation observed is presumptive, but it does gain support by the bone marrow transplantation experiments. It is proposed that either a factor accounting for slower tumor growth is present in old marrow (and that factor may be important in the observed age advantage described previously) or that a factor in young marrow favors tumor growth, and that factor diminishes with aging. Our experiments to date do not provide resolution of this question but do allow speculation.

In all mammalian species, there is an age-related decline in immune function which begins before sexual maturation and develops progressively thereafter (11, 16). This immune deficiency develops coincidentally with the gradual involution of the thymus gland, and thymic-related (or T-cell) immune functions are most significantly involved in immune senescense (1, 4, 8). Age-related immune deficiency is considered by many to at least partially account for the observed high incidence of neoplasia with advancing age (7). Nevertheless, we have observed in these experiments and in our clinical studies (3) that tumor growth is slower and metastases less in older hosts. Can immune deficiency *per se* account for the apparent age advantage once a tumor has developed? Alternatively, is there an immune factor that is enhanced with aging that could explain this seeming paradox? In certain animal models, there is some evidence that immune-deficient hosts have slower growth and fewer metastases than immune-intact controls (5), whereas in others, immune deficiency correlates with more aggressive courses (13). This curious discrepancy is of great interest and importance for biologists studying immune senescense.

One mechanism by which immune deficiency could result in more favorable tumor restraint is if the deficiency involves, to a disproportionate extent, suppressor cell function. T-suppressor cells have been demonstrated in experimental tumor models to develop coincidentally with tumor growth and to specifically undermine host tumor control mechanisms (6, 9). Such inappropriate suppressor cell activity may indicate this mechanism as one in which otherwise immune-competent animals may escape

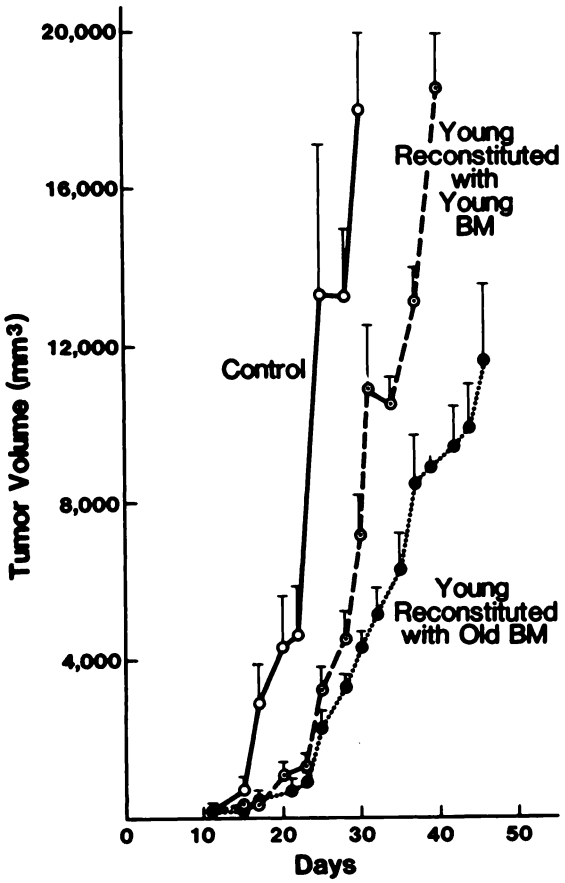

Chart 5. B16 melanoma growth in lethally irradiated young mice reconstituted with either young or old bone marrow (*BM*). Mice (2 to 3 months old, 20/group) received 950 rads total body radiation, and 1 hr later, syngeneic transplantation of bone marrow from either young (2 to 3 months) or old (24 to 25 months) donors. One month later, 10^6 B16 melanoma F_{10} cells were injected s.c. in the right flank. Local tumor growth was measured twice weekly, and the results for each group were depicted. Tumor growth was fastest in controls, intermediate in mice reconstituted with young marrow, and slowest in mice reconstituted with old marrow. After 30 days, the difference in tumor volumes between mice reconstituted with young or old marrow reached statistical significance ($p < 0.05$) for that and each subsequent day.

immune surveillance (1). In aged animals known to be deficient in other T-cell functions, disordered immunoregulation has been described (8, 11, 16, 17). Associated with immune senescense, a decreased capability for tumor-induced, T-cell-mediated immune suppression may offer explanation for the observed age advantage. Alternatively, transplantable cellular or soluble factors that favor tumor growth may be present to a greater extent in young bone marrow. An example of such a factor(s) would be lymphocyte- or macrophage-produced angiogenesis factor. Inasmuch as these are lymphokines or monokines, reduced levels associated with advanced age would not be an unreasonable expectation (11). Indeed, B16 melanoma grown in old animals appears less vascular by standard histological evaluation (light microscope) (4).

The greater number of pulmonary colonies observed in irradiated mice could also reflect immunodeficiency, noting that, in other immune-deficient states, pulmonary colonization has been shown to increase (10, 18). The damaging effects of radiation on the pulmonary infrastructure, however, could also account for the increase. Similar pulmonary endothelial toxins, such as high oxygen concentrations (14), or the anticancer drugs cyclophosphamide and bleomycin (15) have been associated with increased colonies after i.v. injection as well. Additionally, it should be commented on that i.v. tumor cell injection is different from spontaneous metastases, and the conclusion regarding metastases from these and similar experiments is tenuous.

Clearly, further studies will be required to establish with certainty those host factors responsible for the apparent age advantage in these tumor models. Resolution of these factors may prove to be important for our further understanding of both tumor and aging biology.

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