Role of the Thymus and T-Cells in Slow Growth of B16 Melanoma in Old Mice

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ABSTRACT

Clinical observations suggest that tumors grow more slowly in aged subjects. To investigate the influence of age on tumor growth, we injected the same number of cultured B16 melanoma cells into C57BL/6 mice of various ages. B16 melanoma cells, inoculated s.c., grew more slowly in old (18–20-month-old) compared to young (6–8-week-old) mice. In young tumor-bearing mice there was a significant increase in the number and the proliferative response to phytohemagglutinin and concanavalin A of splenic T-cells as compared to old tumor-bearing animals. There was no difference in the response of B-lymphocytes from old and young tumor-bearing mice to lipopolysaccharide. The positive association between T-cells and the rate of tumor growth was also suggested by the slower growth of melanoma cells in thymectomized or thymectomized and anti-α antiserum-treated young mice. Finally, the age-associated difference in tumor growth could be transferred by spleen cells from old or young mice to thymectomized and lethally irradiated syngeneic young animals. Young mice with rapidly growing B16 melanoma tumors have increased numbers and proliferative responses of thymic-derived lymphocytes. It is likely that T-cells or their products facilitate the growth of B16 tumor cells.

INTRODUCTION

The incidence of malignant tumors increases progressively with age in humans (1) and experimental animals (2, 3). Although there is a higher frequency of malignant tumors in the aged, their growth has been reported to be slower (5–7). To investigate whether immune senescence (8–11) is one of the factors that plays a role in the reduced growth of malignant tumors in old animals, the slower rate of growth of the B16 melanoma in old C57BL/6 mice (5) was studied. We have found that the characteristic rate of tumor growth in old and young mice is correlated with the number and functional competence of their T-cells. These findings suggest that the loss of thymic-dependent immunity with age may contribute to the slower growth of tumors in old animals.

MATERIALS AND METHODS

Cells. The F10 subline of the B16 murine melanoma has been maintained in the laboratory of Dr. W. B. Ershler (University of Wisconsin, Madison, WI). Cells were stored frozen in 90% FCS and 10% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO). The cells were grown in Dulbecco's modified Eagle's medium (KC Biologicals, Lenexa, KS) supplemented with 10% heat-inactivated FCS (GIBCO, Chagrin Falls, OH), 2 mM glutamine, and antibiotics. Semi-confluent monolayers were dispersed using 0.25% trypsin (GIBCO, Grand Island, NY) and suspended to 2.5 x 10^6/ml in phosphate-buffered saline for inoculation.

For inoculation with B16 melanoma cells grown in vivo, a tumor 7–10 mm in diameter was aseptically removed from each of either 3 old or 3 young mice and teased apart with forceps. Single cell suspensions were obtained by filtration through sterile gauze. The cells were suspended at a concentration of 5 x 10^6 cells/ml in phosphate-buffered saline.

Mice. Young (6–8 weeks) and old (18–20 months) C57BL/6 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Old mice were housed under laminar flow conditions on arrival from the supplier to safeguard the health of these delicate and rare resources. After tumor inoculation all mice were maintained under flow conditions. All animals received Ralston-Purina Chow No. 5008.

Thymectomy. Thymectomy was performed when mice were 6–8 weeks old. Mice were anesthetized with tribromoethanol and the thymus was removed by suction through a glass cannula through a midline longitudinal incision in the sternum.

Treatment with Anti-α Antiserum. For depletion of the peripheral T-cells, anti-α antiserum was prepared by immunizing rabbits with CBA/J mouse brain in complete Freund's adjuvant as described by Golub (12). This antiserum killed thymocytes and eliminated the in vitro response of spleen cells to PHA. The response to LPS was unaffected. Eight weeks after thymectomy, the young thymectomized mice were treated twice with i.v. injection of 0.2 ml of either Millipore sterilized rabbit anti-α antiserum diluted 1:4 in phosphate-buffered saline or similarly treated normal rabbit serum 3 days apart. B16 melanoma cells were inoculated 4 days after the second injection.

Irradiation and Cell Transfer. In some experiments, 8 weeks after thymectomy, young thymectomized mice were exposed to 850 rads γ-irradiation from a Gammarator M (Radiation Machinery Corp., Parsippany, NJ) and given i.v. injections of 10 x 10^6 spleen cells from young or old syngeneic mice. These mice were then housed under laminar flow conditions. This dose of radiation has been found to be 100% lethal for C57BL/6 mice, unless the irradiated mice were reconstituted with spleen cells.

Inoculation of B16 Melanoma Cells. Mice were given s.c. injections into the shaved right flank of 5 x 10^6 B16-F10 cells grown in vitro or 10 x 10^6 B16-F10 cells grown in vivo in a total volume of 0.2 ml. Tumor growth was examined daily and palpable tumors were measured in 2 perpendicular axes with tissue calipers and tumor volume was calculated assuming spherical growth using the formula 4/3 π r^3.

Flow Cytometric Analysis of Spleen Cell Subpopulation. Spleen cells were filtered through gauze and erythrocytes lysed with Tris-ammonium chloride buffer. Cells were washed and resuspended to 10^6 cells/ml in RPMI 1640 containing 5% FCS and 0.1% sodium azide. Cells (10^6) in 0.1 ml of medium were incubated with saturating concentrations of fluorescein-conjugated anti-Thy-1-1.2 monoclonal antibody (Becton Dickinson, Mountain View, CA) or fluorescein-conjugated goat anti-mouse immunoglobulin antibody (IgG fraction) (Cappel Laboratories, Cochranville, PA) for 30 min on ice. Cells were washed and resuspended in 0.5 ml of medium for analysis of fluorescence on an Ortho Cytoflowgraph System 50H interfaced to a 2150 computer. From 5,000 to 10,000 cells were counted for each sample.

Culture of Spleen Cells. Spleen cells were suspended in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and antibiotics. Cells (2 x 10^6) were cultured in sterile multiwell round-bottomed microwell culture plates (Linbro Chemical Co., New Haven, CT) in a total volume of 0.2 ml containing 2.5 μg/ml PHA (Wellcome, Beckenham, England), Con A (Sigma), or LPS (Difco, Detroit, MI). Cultures were incubated at 37°C for 48 h. [3H]Thymidine incorporation was measured during the last 8 h of culture by adding 1 μCi of [3H]thymidine (specific activity, 2 Ci/mmol; Amersham Corp., Arlington Heights, IL).

Statistical Analysis. Experimental groups were compared statistically using the unpaired Student's t test. The mean ± SE is given.
RESULTS

Slower Growth of B16 Melanoma Cells in Old Compared to Young Mice. B16 melanoma cells (5 x 10⁵) were inoculated into young (6-8-week-old) or old (18-20-month-old) male C57BL/6 mice. The growth of B16 melanoma was significantly slower in old male mice compared to young male mice (Fig. 1) confirming previously reported observations (5). For example, 18 days after tumor inoculation, tumor size in old mice was 21% of that in young mice. We also found that the growth of the B16 melanoma was significantly slower in old female mice compared to young female mice. Thus, in females, 18 days after inoculation, tumor size in old animals was 14.1% of that in young mice. In all subsequent experiments male mice were used.

Comparison of Growth Potential of B16 Melanoma Growing in Young and Old Mice. To determine if the age of the host resulted in selection of subpopulations of tumor cells having different growth rates, young and old mice were given injections of B16 melanoma cells which had been grown in old or young mice instead of in culture. The rate of tumor growth was rapid in young but slower in old animals and was not influenced by the prior residence of the cells (Fig. 2). Thus, the observed difference in the rate of growth of the B16 melanoma cells in old and young mice does not depend upon selection of subpopulations of melanoma cells by the host but upon the capacity of the host animal to support tumor growth.

Effect of B16 Melanoma Cells on the Number and Function of Spleen Cells. Spleens were removed from young, young thymectomized, and old mice when the tumor size was about 1 cm in diameter and the number of spleen cells (Table 1) and their responses to PHA, Con A, and LPS were determined (Table 2). The total number of spleen cells was significantly greater in young tumor-bearing mice (136 x 10⁶) than in tumor-bearing young thymectomized mice (73 x 10⁶), in old tumor-bearing mice (74 x 10⁶), or non-tumor-bearing old (77 x 10⁶) or young (82 x 10⁶) mice. The presence of the B16 tumor was associated with an increased number of spleen cells in young intact mice but not in thymectomized young or intact old mice. Efforts were made to determine the type of cell responsible for the increased number of spleen cells in young tumor-bearing mice. Melanoma cells were easily distinguished microscopically from splenic lymphocytes and macrophages. No melanoma cells were detected in spleen cell suspensions from melanoma-inoculated mice. As seen in Table 1, the absolute number of splenic T-cells was significantly greater in melanoma-inoculated mice, while the number of cells which reacted with fluorescent anti-immunoglobulin antisera (B-cells and some macrophages) was not different in melanoma-inoculated and non-tumor-bearing young mice. There was also a significant increase in

![Graph](image)

**Fig. 1.** Effect of age of host on the rate of growth of B16 melanoma tumors. B16 melanoma cells (5 x 10⁵) were inoculated s.c. into young or old male C57BL/6 mice. Tumor size in two dimensions was measured and the volume of tumor was calculated. Data are presented as means for groups of 6 mice. Bars, SE.

**Fig. 2.** Melanoma cells grown in vivo are comparable to those grown in vitro. Single cell suspensions from B16 melanomas growing in young or old mice were prepared. The tumor cells (10⁶) from young mice were inoculated into young mice and the tumor cells from old mice were inoculated into young or old mice. Data are the mean tumor volume, at the time after inoculation indicated, for groups of 6 mice. Bars, SE. A, tumor cells which had been grown in old mice inoculated into young mice; B, tumor cells which had been grown in young mice inoculated into young mice; C, tumor cells which had been grown in old mice inoculated into old mice.

**Table 1** Effect of B16 melanoma on the spleen cell populations of young C57BL/6 mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Thymectomy</th>
<th>B16 melanoma bearing</th>
<th>PHA</th>
<th>Con A</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>+</td>
<td>23.9 ± 2.2</td>
<td>29.9 ± 3.1</td>
<td>13.9 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>+</td>
<td>25.3 ± 2.9</td>
<td>26.6 ± 2.9</td>
<td>12.4 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>+</td>
<td>15.6 ± 2.2</td>
<td>19.2 ± 2.9</td>
<td>14.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Old</td>
<td>−</td>
<td>8.9 ± 1.3</td>
<td>12.6 ± 2.2</td>
<td>10.8 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Old</td>
<td>+</td>
<td>11.5 ± 0.9</td>
<td>12.9 ± 0.6</td>
<td>14.3 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2** Effect of B16 melanoma on the proliferative responses of spleen cells from old and young mice to PHA, Con A, and LPS

<table>
<thead>
<tr>
<th>Cell population counted</th>
<th>Normal mice</th>
<th>B16 melanoma-bearing mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total spleen cells*</td>
<td>83.8 ± 4.7</td>
<td>136.2 ± 7.6</td>
</tr>
<tr>
<td>Thy-1.2-positive cells*</td>
<td>45.6 ± 3.2</td>
<td>62.6 ± 4.4</td>
</tr>
<tr>
<td>B-Cells and macrophages</td>
<td>37.0 ± 2.2</td>
<td>37.8 ± 3.2</td>
</tr>
<tr>
<td>Other cells</td>
<td>2.5 ± 1.7</td>
<td>25.9 ± 11.7</td>
</tr>
</tbody>
</table>

* Difference between normal mice and melanoma-inoculated mice was statistically significant. a, (P < 0.001); b, (P < 0.02).
in cells not stained by either anti-Thy-1.2 or anti-immunoglobulin antibodies in tumor-bearing young mice. The lineage of these cells, at the moment, is not known.

Not only were there more T-cells in the spleens of young tumor-bearing mice than in either young thymectomized mice or old tumor-bearing mice but the proliferative response of spleen cells to PHA or Con A in tumor-bearing mice was greatest in the young, intermediate in the young thymectomized mice, and lowest in the old mice (Table 2). The rate of tumor growth was correlated with the vigor of the proliferative response of T-cells in tumor-bearing mice. There was, however, no difference in the responses to LPS by spleen cells from old or young normal or tumor-bearing mice (Table 2). In the normal mice without melanoma, the proliferative responses of spleen cells to PHA or Con A was less in old mice than in young mice.

Slower Growth of B16 Melanoma Cells in Young Mice Depleted of Thymic-dependent Cells by Thymectomy and Treatment with Anti-0 Antiserum. To determine whether the age-associated involution of the thymus gland and impaired T-cell function might contribute to the slower growth of the B16 melanoma cells in old mice, we measured the growth of the B16 melanoma cells in young thymectomized mice. B16 melanoma cells were inoculated into young mice which had been sham-thymectomized or thymectomized at 6 weeks of age. In the young thymectomized mice, growth of the melanoma was significantly slower than that in young control mice (Fig. 3). Some young thymectomized mice were also treated with anti-0 antiserum. The growth of the B16 melanoma was even slower in these animals than in young thymectomized mice. The results are consistent with the interpretation that the thymus gland and 0-positive T-cells contribute to the rapid growth of melanoma cells in young mice.

Characteristic Age-associated Tumor Growth Rate is Transferred with Spleen Cells to Thymectomized, Lethally Irradiated Mice. Young thymectomized mice were lethally irradiated and given injections of spleen cells from syngeneic young or old mice. Eight weeks after irradiation, B16 melanoma cells were inoculated into the irradiated recipients of spleen cells from young or old mice (Fig. 4). The growth of B16 tumor cells is slower in irradiated animals. However, the growth of melanoma cells in recipients of spleen cells from old donors was significantly slower than in recipients of spleen cells from young donors. These results suggest that the characteristic age-associated difference in tumor growth is transferable with spleen cells to thymectomized, lethally irradiated young mice.

DISCUSSION

B16 melanoma cells grow more slowly in old as compared with young mice. The mechanism of this phenomenon is still controversial. Although immunological mechanisms have classically been considered to destroy tumor cells, there is considerable evidence that immunological activity may, in some circumstances, augment the growth of at least certain tumors. Reduced tumor growth in old mice might be secondary to the well-known age-related decline in immune responses. The reduced growth of the B16 melanoma in old mice has been reported previously to be transferred to young irradiated recipients by bone marrow from old mice (13). In this report the characteristic tumor growth rate in old or young mice was transferred to lethally irradiated young animals with spleen cells. We have subsequently found, in preliminary studies,4 that it is the T-cells in the transferred spleen cell preparation which determines the rate of B16 tumor growth.

The growth of B16 melanoma was also slower in young thymectomized mice than in young untreated mice and still slower in young, thymectomized, anti-0 antibody-treated mice. The results are thus consistent with the view that the more vigorous T-cell immune responses typical of young animals are responsible for the rapid growth of the tumor in young animals. The increase in splenic T-cells which we observed in young thymectomized mice, and to T-cell mitogens. These results suggest again that decreased immunocompetence, especially decreased T-cell function, may be responsible for the slower growth of the tumor in old mice. It is reasonable to suggest that the immune response

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The text contains a graph and a table, which are not described in the natural text representation.
to melanoma cells might facilitate the growth of the tumor as a consequence of the production of some critical growth factor or factors. In old mice and young thymectomized mice, reduced immunocompetence may result in a loss of activity to the tumor cells and thereby result in less facilitation by T-cells of tumor cell growth than that seen in young mice.

Neovascularization is an essential element of solid tumor growth (14). New vessel growth is stimulated by angiogenic factors which can be generated by tumor cells or by activated T-cells (15) and macrophages (16). It has been reported (5) that B16 tumors growing in old mice are less well vascularized than tumors growing in young mice. This was also observed in tumor specimens obtained in the present studies (data not shown). The reduced vascularity of tumors in old mice could result from the reduced production of angiogenic factors by T-cells in old mice that facilitate vascularization and tumor growth.

Previous workers have suggested that immune responses to tumors could have both positive and negative effects on the tumor growth (17–20). For example, Prehn (17) demonstrated that a small number of specifically sensitized spleen cells mixed with a constant number of sarcoma cells accelerated the growth of the tumor but a large number of these spleen cells inhibited tumor growth in Winn-type assays. Fidler (18) demonstrated a similar biphasic effect of sensitized spleen cells on the pulmonary metastases of B16 melanoma cells.

It is also possible that the growth of B16 melanoma cells in old animals is actively suppressed by humoral or cellular factors. Mouse serum appears to contain factors that inhibit the growth of B16 tumor cells in vitro. We have observed a greater concentration of suppressor activity present in old as compared to young mouse serum.5

Finally, mention should be made of the potential adaptive value of immune senescence. Several years ago, Weksler (21) suggested that the tendency of old animals to form autoantibodies and benign monoclonal immunoglobulins might be accentuated if immunocompetence were increased. The present studies suggest further caution with respect to nonspecific immune enhancement in old animals. It is possible that, in some cases, tumor growth might be increased in aged subjects if their immunocompetence were enhanced. Thus, it is possible that the age-associated reduction in immunocompetence might have a significant survival value to the species. Clearly, the value of such a mechanism would be determined by the incidence of tumors the growth of which is augmented by immune responses and the risks of increased autoimmunity, counterbalanced against the reduced capacity for defense against infectious agents and the reduced ability to destroy certain tumors.

ACKNOWLEDGMENTS

We wish to thank Dorothy Parks for her skillful preparation of the manuscript.

REFERENCES


5 T. Tsuda et al., unpublished observations.
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