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Restoration of Impaired Immune Functions in Aging Animals

V. Long-Term Immunopotentiating Effects of Combined Young Bone Marrow and Newborn Thymus Grafts[1]

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Seventeen-month-old female B6C3F1 mice (mean life span, 30 months) were given 600 R, and grafted with young bone marrow cells and newborn thymic lobes, and their immunologic activities were assessed 11 months later when they were 28 months old. The results revealed that the treated mice were superior to the untreated age control mice and almost comparable to 14-month-old mice, in terms of mitogen response to PHA and Con A, humoral response to sheep red blood cells, and cell-mediated cytolytic response to allogeneic cells. No attempt was made to assess the effect of grafting on life span, as all the mice were used for immunologic studies at 28 months of age. No difference was observed between the treated and untreated mice in their survival time and tumor incidence at the time of sacrifice.

INTRODUCTION

Various immunologic activities decrease with age, as a result of changes occurring primarily in the T-cell compartment of the immune system (1). The changes are caused in part by the inability of age-related involuted thymus to efficiently promote T-cell differentiation (2, 3). Of clinical importance is the decrease in immunologic activities associated with a corresponding increase in the incidence of infectious, neoplastic, and autoimmune (immune complex) diseases (1, 4–6). Correlational and drug therapy studies indicate that the events are causally related and, moreover, the decline in immunologic activities is responsible for the increase in the incidence of diseases (4–9). It would seem desirable, therefore, to explore methods of preventing or reversing the declining immunologic activities of aging individuals because a successful method could be used to determine the extent to which potentiation of immunologic activity can alter the onset and/or incidence and severity of age-related immunologic disorders. It could also be used as a probe to identify cells which are vulnerable to aging.

In our initial attempt to potentiate immunologic activities of long-lived aging mice, middle-aged mice were lethally X irradiated and grafted with either young bone marrow cells, newborn thymus, or young marrow cells plus newborn thymus (10). The combined young bone marrow and newborn thymus grafts were found to be superior to either alone, supporting the view that both the precursor T cells (11) and thymic stroma, the generators of T-cell differentiating factors, are affected by

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aging (1–6). Moreover, the magnitude of increase in immunologic activities of aging mice receiving the combination grafts was found to be quite substantial when tested 3–4 months after grafting, as the level of activities approached or even exceeded that of adult mice. Thus, the next issue that needs to be resolved is whether or not their effect on immunologic activities is long lasting. Accordingly, the present study was attempted to assess the long-term immunologic effects of the combined young bone marrow and newborn thymus grafts in long-lived aging mice.

MATERIALS AND METHODS

Mice. Long-lived female (C57BL/6 × C3H) F1 mice (mean life span, 30 months), hereafter referred to as B6C3F1 mice, were obtained from the Animal Production Facility at National Institute of Radiological Sciences, Chiba, Japan.

Treatment. Forty-six mice were divided equally into the age control and experimental groups in a random manner, when they were 17 months of age. The experimental mice were exposed to 600 R of X ray (200 KVP, 20 mA, added filtration of 0.5 mm Cu plus 0.5 mm Al, target-skin distance of 50 cm, 100 R/min in air). A dose of 600 R was chosen over 850 R, the dose employed in our previous study (10), because preliminary studies on radiation dose–bone marrow transplantation take revealed that they were comparable in providing a milieu for a long-lasting take of bone marrow grafts. Immediately after irradiating the mice, they were injected intravenously with 2×10^6 bone marrow cells from 2-month-old syngeneic mice. A month later, two thymic lobes from newborn syngeneic mice were grafted under the capsule of their right kidney (3). Two out of twenty-three mice of the treated group died within a week. Beginning a month after thymic grafting when the mice were 19 months old, both groups of mice were inspected daily for survival 6 days a week until they were 28 months old. The mice were then killed for immunologic assessment.

Antibody response. Antibody response was assessed against sheep red blood cells (SRBC) by injecting intraperitoneally 3×10^8 SRBC, and 4 days later the mice were killed and their spleens were dispersed and assessed for direct anti-SRBC plaque-forming cells (DPFC) according to the method of Plotz *et al.* (12). Spleen cells of individual mice were also assessed for their ability to mount an *in vitro* mitogenic and cell-mediated cytolytic response, since our previous study (10) and subsequent confirmatory preliminary study had shown that injection of SRBC 4 days beforehand had no influence on the *in vitro* response of spleen cells to mitogenic and allogenic stimulation.

Mitogenic response. Mitogenic response was assessed in triplicate wells in microplates (Falcon 3042) containing 5×10^5 spleen cells in 0.2 ml of RPMI 1640, supplemented with 5% fetal calf serum and 12 μg kanamycin. Cells were stimulated with an optimum dose of either phytohemagglutinin (PHA, Wellcome Reagent Ltd., England, 1 μg), concanavalin A (Con A, Sigma, St. Louis, Mo., 1 μg) or *E. coli* lipopolysaccharide (LPS, Difco, Detroit, Mich., 1 μg), as determined in preliminary studies and incubated at 37°C in 5% CO_2 in air atmosphere. Sixty-six hours later, the cultures were pulsed with 0.25 μCi of [^{3}H]thymidine (sp act, 6.0 Ci/mmol) for 6 hr. The cells were recovered with a cell harvester (Model 101,

Labo Science, Tokyo) and processed for liquid scintillation counting (LS-250, Beckman).

Cell-mediated cytolysis response. This was determined by stimulating quadruplicate cultures of 5×10^6 spleen cells of multiwell plates (Nunc-1483) with an equal number of irradiated (1500 R) DBA/2(H-2^d) spleen cells in a 1-ml volume of RPMI 1640 medium, supplemented with 5×10^{-5} *M* 2-mercaptoethanol and 60 μg/ml of kanamycin. The plates were cultured at 37°C in 5% CO_2 in air atmosphere for 5 days, and the cells were then harvested and tested for their cytolytic activity according to the method of Cerottini *et al.* (13). Briefly, 4×10^4 of ^{51}Cr-labeled target tumor cells (L-5178Y,H-2^d) were mixed with varying numbers of spleen cells in a total volume of 0.2 ml of RPMI 1640 medium supplemented with 5% fetal calf serum in microplates (Falcon 3042). The plates were then incubated at 37°C in 5% CO_2 in air atmosphere for 6 hr, centrifuged at 600*g* for 20 min, and 0.1 ml of the supernatant carefully aspirated from each well into a plastic capsule (No. 00, BEEM) for measurement of radioactivity (JDC-751, Aloka, Japan). Percentage specific ^{51}Cr release was calculated from the formula described by Cerottini *et al.* (14), taking into account spontaneous ^{51}Cr release, as measured with target cells incubated alone, and maximal release, as measured with target cells incubated in 0.1% Triton X-100:

$$\% \text{ release} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100.$$

For each sample, a dose—response curve was established and the number of lytic units (LU) calculated. One lytic unit was defined as the number of spleen cells required to lyse 50% of the 4×10^4 ^{51}Cr-labeled target cells within 6 hr.

Statistical analysis. For comparison of population means, Student's *t* distribution program was employed. Regarding DPFC values, they were log transformed to normalize their distribution (15) before statistical analysis.

RESULTS

Survival

Determination of the survival patterns of treated and untreated mice began 1 month after grafting when the mice were 19 months old until 9 months later when the mice was 28 months old. Minimal differences were observed between the two groups. Thus 11 of 21 mice in the treated group (52.4%) and 12 out of 23 in the untreated group (52.2%) were still alive at 28 months old.

Histopathology

Only mice which were killed at 28 months of age were examined histopathologically for malignant tumors. Those dying naturally during the 9-month period of observation were excluded because post-death autolysis was extremely variable. The histopathological results revealed that no difference exists between the two groups, for there were 2 cases out of 11 mice in the treated group with tumors (one Dunn type A splenic reticulum cell sarcoma and one hepatoma) and 3 cases out of

12 mice in the untreated group (two Dunn type A splenic reticulum cell sarcoma and one breast cancer).

Immunologic Activity

a. Antibody response. Treated old mice had a better antibody response to SRBC as measured by DPFC than untreated old mice ($P < 0.05$) (Fig. 1). Thus the responses of 10 out of 11 treated old mice were higher than the mean response of untreated old mice and the response of only 1 out of 12 untreated old mice was higher than the mean response of the treated old mice. However, it should be noted that the mean responses of both the treated and untreated old mice were an order of magnitude lower than that of untreated adult and young mice.

b. Mitogenic response. The LPS response, reflective of the proliferative capacity of B cells, was not affected by age (i.e., between 4 to 28 months of age) and by treatment of aging mice (Fig. 2). On the other hand, the combined young bone marrow–newborn thymus grafting had a marked immunopotentiating influence

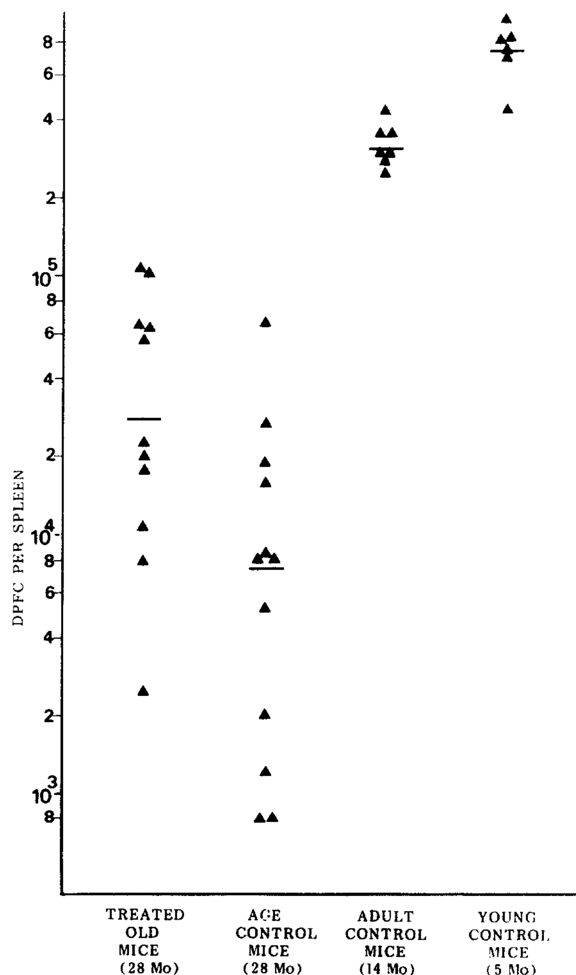

FIG. 1. Day 4 anti-SRBC plaque-forming cell response. Horizontal bars show mean response levels.

on spleen cells of old mice in their proliferative response to PHA and Con A stimulation. Thus, the proliferative response of spleen cells from treated old mice was apparently superior to that of untreated old mice ($P < 0.05$) and almost comparable to that of untreated adult mice. It should also be noted that the low response of untreated old mice was not due to the presence of the tumor, since untreated old mice without tumor also showed low response.

c. Cell-mediated cytolysis response. The combined young bone marrow–newborn thymus grafting also had a long-lasting immunopotentiating effect on the cytolytic T-cell response against allogeneic target cells (Fig. 3). Thus, the activity of spleen cells of treated old mice was higher than that of untreated old mice ($P < 0.05$) and untreated adult mice, although still lower than that of untreated young mice.

DISCUSSION

Earlier studies on grafting in aging animals focused on life extension rather than on immunopotentiation, and they revealed that, with one exception, the effects were marginal regardless of whether the aging mice were long lived, autoimmune resistant or short lived, autoimmune susceptible (16–19). The exception was observed by Fabris *et al.* (20), who found that injection of a large dose of syngeneic lymph node cells into short-lived, growth hormone-deficient hypopituitary dwarf mice resulted in a three- to fourfold extension of life span. It is of interest that only

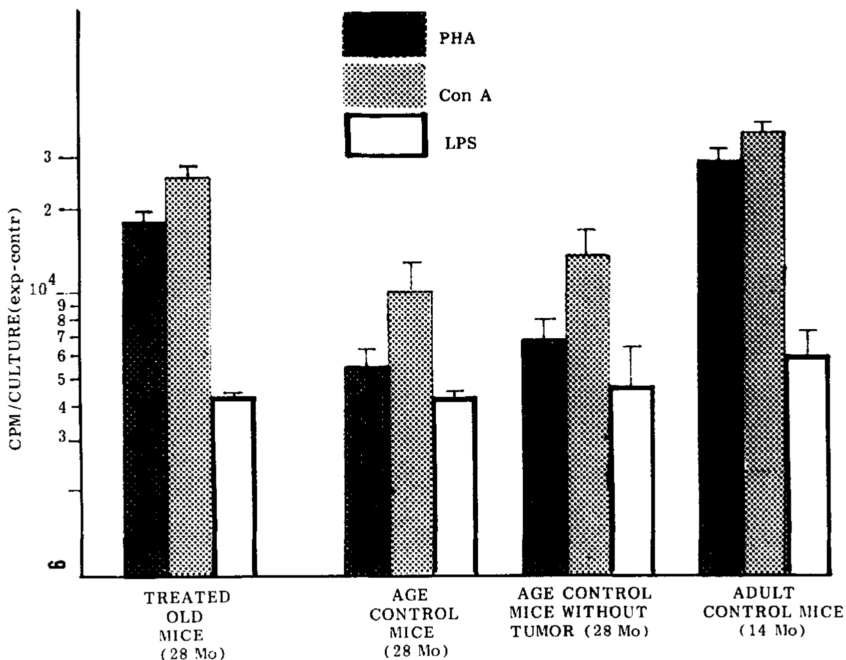

FIG. 2. Mitogenic response of spleen cells to phytohemagglutinin (PHA), concanavalin A (Con A), and lipopolysaccharide (LPS). Vertical bars, one standard error of the mean. Number of mice examined: 10 treated old mice, 11 age control old mice, 8 age control old mice free of tumor, and 3 age control adult mice.

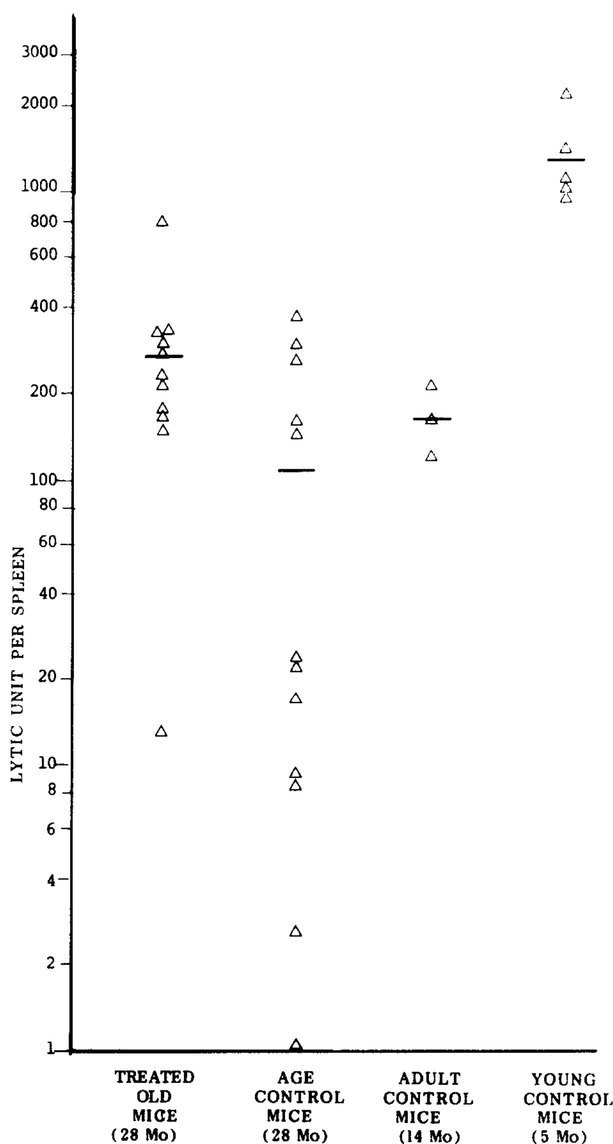

FIG. 3. Day 5 cell-mediated cytolytic response. Horizontal bars show mean response levels.

one type of tissue was employed in these grafting studies; i.e., either thymus, bone marrow, spleen, or lymph node cells.

Based on the experience of earlier investigators, we felt that three criteria must be met for a graft to be effective in modulating age-related diseases and perhaps the life span, as there are many factors responsible for the decline with age in immunologic activities. (i) More than one type of tissue may be needed to be included in the graft. (ii) The potentially deleterious cellular environment of the host needs to be minimized to enable the graft to flourish maximally. (iii) The graft must have a long-lasting immunopotentiating effect. Accordingly, a study was

initiated to resolve the first two criteria (10). The first was approached by assessing the effectiveness of simultaneous newborn thymus and young bone marrow grafts because previous studies revealed that the T-cell compartment of the immune system is most vulnerable to aging (1–6, 11). The second was approached by exposing prospective recipient aging mice to a total body X irradiation to destroy their radiosensitive cells and at the same time provide a cellular environment conducive for maximal growth of the grafts. The results showed that the combined newborn and young bone marrow grafts when transplanted into preirradiated middle-aged mice were most effective in promoting immunologic vigor.

The present study was carried out in an attempt to resolve the third criterion; i.e., whether the combined newborn thymus and young bone marrow grafts when transplanted into preirradiated middle-aged mice have a long-lasting immunopotentiating effect. The results presented here show that this grafting protocol is indeed very effective in providing a high level of T-cell-dependent immunologic activity in aging mice for a long period of time. Thus, 10 months after grafting, which is 0.34 of a mean life span or equivalent to about 25 human years, when the mice were 28 months old, the T-cell proliferative response was superior to that of the untreated age-matched control mice and almost comparable to that of the untreated adult mice. The cell-mediated cytolytic T-cell response was also elevated and superior to that of untreated age-matched control mice and of untreated adult mice. On the other hand, the T-cell-dependent antibody response was elevated to a lesser degree proportionately, as it was only slightly superior to that of untreated age-matched control mice and was an order of magnitude lower than that of untreated adult and young mice. In this regard, it should be noted that the level of antibody response observed 4.5 months after grafting in our previous study was very high (10), in contrast to that observed 10 months after grafting. These results would suggest that the grafts are succumbing slowly to the radioresistant host factors which are promoting suppressor cell activities and/or impeding helper activities.

The effect of grafting on life span and disease incidence could not be assessed, as all the mice were sacrificed for immunologic studies at 28 months of age. However, at the time of sacrifice the improved immune potentials in old mice were associated with neither a longer life span nor decrease of tumor incidence. It could be that the mice employed here are naturally long lived with a low incidence of spontaneously occurring tumor. If so, a better animal model to assess the influence of this grafting protocol on disease pattern and life span would be shorter-lived mice which are highly vulnerable to cancerous or autoimmune diseases.

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