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Long-Term Effect of Whole-Body X-Irradiation on Cell-Mediated Immune Reaction in Mice

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Age-related change in immunological activity was examined at 10 to 91 weeks following whole-body irradiation by determining the specific anti-tumor cell-mediated immunity in host mice induced and/or enhanced by local irradiation to transplanted tumor. Median survival time of the non-irradiated C3H/He female mice was 98.6 weeks while the median life-span of the mice exposed to two and four Gy of 250kVp X-rays at the age of 10-12 weeks was shortened by 14.9 and 23.4 weeks, respectively. The rate of tumor reduction within two weeks after local irradiation to tumor and the growth inhibitory activity of spleen cells from tumor irradiated mice were reduced in a dose-dependent manner when assessed 10 weeks after whole-body irradiation, but recovered to the near-complete level of the non-irradiated controls within a few months, then gradually decreased with normal aging. These results suggest that the age-dependent decline of this immunological activity appears earlier in the irradiated mice as a result of whole-body X-irradiation at a young age, suggesting accelerated aging of the immune system.

INTRODUCTION

Studies using *in vivo* animal models have made important contributions to our understanding of radiation-induced life-shortening and radiation carcinogenesis. However, the hypothesis that radiation accelerates natural aging is today far less attractive than it was a few decades ago. Ionizing radiation does, of course, shorten animal and human life, but its life-shortening effect appears to be due to the increased incidence of malignant tumors at a low dose range of low-LET radiation[1-3].

It is now well established that immune functions decline with advancing age by causing deleterious changes in the lymphoid system[4] and that the lowered immune functions may play an important role in the higher risk of the aged to infectious disease, autoimmune disorders as well as malignant tumors[4-6]. The immunosuppressive effects of irradiation have also been well

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documented by Anderson *et al.*[5] and others[6-8]. Furthermore, the late effects of irradiation on the immune system of mice has been studied using many immunological parameters, but the accumulated results vary considerably depending on the assay system, mouse strain and mouse maintenance conditions as well as the age of mice at the time of whole-body irradiation[6]. In fact, some researchers reported that whole-body exposure of mice to sublethal doses of radiation during early life caused accelerated aging in the immunological functions when assessed by antibody response to sheep red blood cells (SRBC)[9,10], graft-vs-host reaction[11], mixed lymphocyte reaction[12] and mitogen responsiveness[13] as a measure of immunological activity of aging mice, while others reported that it did not cause accelerated aging when assessed by primary antibody response of intact mice to SRBC, mitogen responsiveness of spleen cells *in vitro*, and cytotoxic T cell response of spleen cells *in vitro*[6,12].

Because of the diversity of late effects reported in the literature, we have investigated the long-term effects of whole-body X-irradiation by employing a novel method that allows assessment of anti-tumor immunologic function. The basic principle of this method is to assess the role of spleen cells in aiding the suppression of growth of transplanted tumor cells following local irradiation to tumor. It is known that local irradiation to a transplanted tumor mass does not kill 100% of the tumor cells. A significant fraction of the tumor cells survive the radiation exposure. However, the growth of the surviving cells seemed to be considerably inhibited by the host immune response initiated by the irradiated tumor tissue[5,14-17]. The authors have previously reported[18] that the specific anti-tumor cell-mediated immunity in host mice could be induced and/or enhanced by local irradiation to tumor of the tumor bearing mouse, and that the spleen cells from these mice inhibited the growth of tumor cells in mice as well as in a culture system. This enhancement of the specific anti-tumor cell-mediated immunity in host occurred one week after local irradiation to tumor, continued for several weeks[18], and depended on the cooperation of non-killer T cells and macrophages[19]. The present investigation was undertaken in order to clarify whether or not radiation exposure during young adult life results in accelerated aging of this type of cell-mediated immune response using inbred C3H/He female mice.

MATERIALS AND METHODS

Animals

Five-hundred eighty C3H/He female mice six weeks old were obtained from Funabashi Experimental Animal Company Ltd. (Funabashi, Japan) and observed for four weeks during which they were checked for body weight and infections. All mice (10 or less per cage) were kept in a 24 ± 2°C air-conditioned clean room and given free access to food and tap water.

X-irradiation

Mice were randomly distributed into three groups, the non-irradiated controls (160 mice) and two irradiated groups exposed to two (180 mice) and four Gy (240 mice) of whole-body irradiation at the age of 10 to 12 weeks. The whole-body X-irradiation of the mice was done under an unanesthetized condition in a special plastic box using a Toshiba EXS-300-4 X-ray

machine (Toshiba Co. Ltd., Tokyo, Japan). The X-irradiation factors were 250kVp, 12mA, 0.5mm Cu + 0.5mm Al filter, 50cm FSD and dose-rate 0.45Gy/min. The control group was sham-irradiated.

Tumor cells

MAC tumor cells were newly established by the authors from a spontaneous mammary tumor MM46 line in C3H/He strain mice[18]. The cells are able to grow in ascites and solid tumor in C3H/He mice and in suspension culture and to form colonies on soft agar. The MAC tumor was passaged as ascites in syngeneic C3H/He mice, and was also cryopreserved in RPMI 1640 medium containing 15% FCS and 10% dimethyl sulfoxide in liquid N_2. For each experiment, frozen MAC tumor cells were thawed, washed, and resuspended in PBS. One hundred thousand cells were injected i.p., and after two to four i.p. passages, tumor cells in ascites were harvested one week later for inoculation into the thigh of mice. The cells were also maintained in suspension culture in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (same supplier), penicillin (100 units/ml) and streptomycin (100 μg/ml), for *in vitro* experiments.

Inoculation of tumor-bearing mice and local irradiation of tumor

The age-related changes in immunological activity were examined at frequent intervals, i.e., 5 to 6 time points, between 10 and 91 weeks following whole-body irradiation, as shown in Fig. 1. For all experiments, healthy test mice, in which there were no neoplastic or infection diseases, were randomly drawn from each of the three populations (i.e., controls and two irradiated groups). The MAC cells from ascites were washed in PBS and subcutaneously injected into the left thigh of C3H/He mice at 10^6 cells/mouse in 0.05ml. One week after the inoculation, for some experiments, the tumors in half the mice from each group were irradiated locally with 20 Gy of 250 kVp X-rays. The whole body except the left leg was shielded by a 5-mm lead plate box.

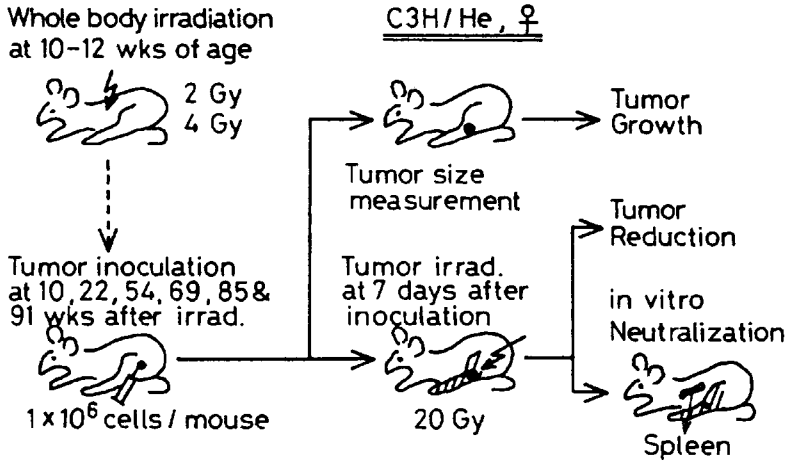

Fig. 1. Scheme of the experimental procedure.

Tumor measurements

Two perpendicular diameters of tumors in each group of mice were measured with vernier calipers in millimeters at three or four-day intervals after inoculation, and the relative size was expressed as their product. Tumor growth rate after inoculation of tumor cells in the left leg was calculated by the following formula:

Tumor growth rate

$$= \frac{\text{relative size of tumor at 14 days after inoculation}}{\text{relative size of tumor at 7 days after inoculation.}}$$

The rate of tumor reduction within two weeks after local irradiation to tumor was calculated as follows:

Tumor reduction rate

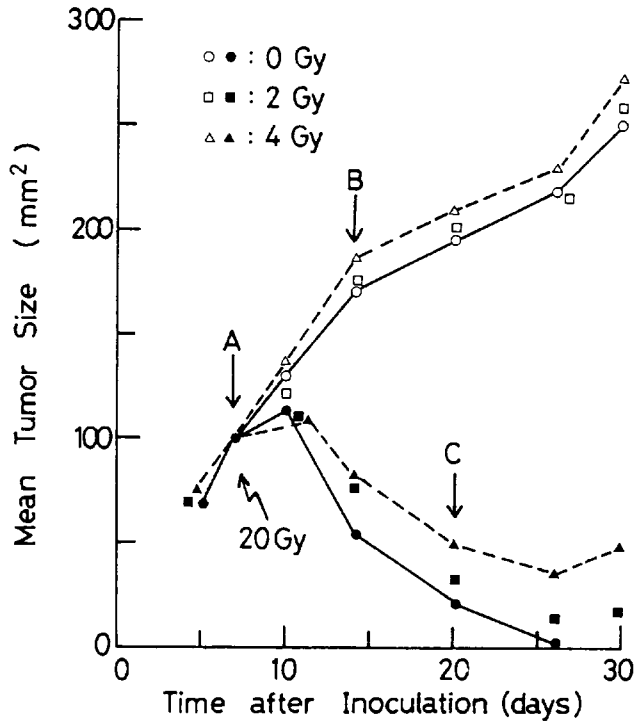

Fig. 2. Growth curve of MAC solid tumor after subcutaneous inoculation of 10^6 cells/mouse at 21 weeks of age in untreated (open symbols) and treated mice (closed symbols) whose tumor in the left thigh was irradiated locally with 20 Gy of X-rays 7 days after inoculation. The tumor growth rate and tumor reduction rate were calculated as follows: Tumor growth rate = (relative size of tumor at 14 days after inoculation (B) / relative size of tumor at 7 days after inoculation (A)). Tumor reduction rate = (1−relative size of tumor at 2 wks after tumor irradiation (C) / relative size of tumor at the time of local irradiation (A)).

$$= 1 - \frac{\text{relative size of tumor at 2 weeks after tumor irradiation}}{\text{relative size of tumor at the time of local irradiation.}}$$

The result of a typical experiment is shown in Fig. 2.

Assay of cell-mediated immune response of spleen cells in vitro

The protocols for determination of growth inhibitory activity of spleen cells from tumor irradiated mice have been described elsewhere[18]. Briefly, spleens aseptically removed from mice, were placed in RPMI 1640 medium, teased to release all of the spleen cells, then gently pushed through a fine stainless steel mesh sieve to obtain single-cell suspensions. After centrifuging supernatants, spleen cells were resuspended in complete culture medium (RPMI + Pen-Strep + 10% FCS) at a concentration of 10^7 cells/ml and one ml of the spleen cell suspension in 35-mm diameter dishes was mixed with one ml of the MAC tumor cell suspension (E/T = 200:1). The mixtures of 1×10^7 spleen cells and 5×10^4 tumor cells in 2 ml of complete culture medium were allowed to undergo cell-mediated immune reaction in these dishes at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for two days. On day 2, they were subcultured (1:10) in complete culture medium and a portion of the cells were further diluted and plated at cloning densities in 0.3% soft agar culture medium. The number of MAC tumor cells in mixed suspension cultures were measured by size distribution analysis four days after being subcultured. For cloning efficiency determinations, the cultures were incubated for 10 to 14 days and the colonies (discrete groups of more than 50 cells) were counted. When the tumor cells were incubated with normal spleen cells, the cloning efficiency of MAC tumor cells ranged from 50% to 85% (majority 60—70%). The growth inhibitory effect of spleen cells was calculated from the following formula: Inhibition activity

$$= 1 - \frac{\text{number (or cloning efficiency) of tumor cells incubated with immune spleen cells}}{\text{number (or cloning efficiency) of tumor cells incubated with normal spleen cells.}}$$

Values obtained from both growth kinetics and colony formation in soft agar are quite similar (data not shown).

RESULTS

The corrected cumulative per cent survivals as functions of age are shown in Fig. 3. Comparison of the slopes of per cent survival in the three groups (non-exposed, 2Gy, 4Gy) shows a systematic displacement to the left consistent with an increasing response to increased dose. Median survival time of the non-irradiated control group was 98.6 weeks and those of the irradiated groups exposed to whole-body irradiation of two and four Gy at the age of 10—12 weeks were 83.7 and 75.2 weeks, respectively. Although this investigation did not provide information on the qualitative differences between diseases arising in these groups, spleen and body weights were measured for individual mice of each group in some experiments. The mean values of spleen weight increased with age from 0.18 ± 0.02 (21 weeks of age) to 0.25 ± 0.07 (96 weeks of age)

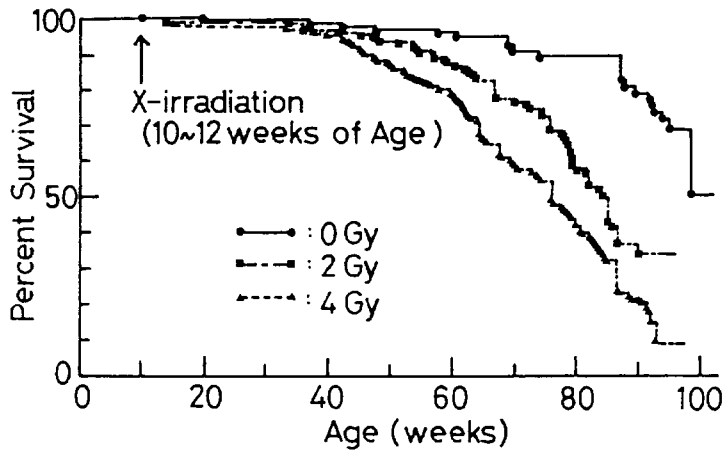

Fig. 3. Percent survival with age in controls (●——●) and whole-body irradiated mice (■—·—■; 2 Gy, ▲----▲; 4 Gy). Cumulative survivals were calculated by the method of Kaplan-Meier. Significant differences in median survival time were seen among the three groups at the 1% level.

in the control group. The body weight also increased with age. There were, however, no significant differences in any these values among the three groups.

When 1×10^6 tumor cells were inoculated into the left thigh of young control C3H/He mice, the tumor grew to approximately 10-mm in diameter at 7 days after inoculation; the relative size of tumors increased 1.4-fold during an additional week as shown in Fig. 2. Changes in the tumor growth rate of control and whole-body irradiated groups with age are shown in Table 1. The changes in tumor growth rate were approximately linearly related to age over the age interval between 33 and 102 weeks in the control group. At the age of 21 to 96 weeks, although there was high correlation with age ($p < 0.01$) for the control group, the difference in tumor growth rate between whole-body irradiated and non-irradiated age-matched control mice was not significant at the 5% level.

One week after inoculation, the tumors in half the mice from each group were locally irradiated with 20 Gy of X-rays. Tumors in young control mice were markedly reduced and disappeared within three weeks after local irradiation to tumor (Fig. 2). The rate of the tumor reduction within two weeks after tumor irradiation was about 80% for young control mice, gradually decreasing from 65 to 102 weeks of age in the control group (Table 1 and Fig. 4). In contrast, the tumor reduction rates of the irradiated mice were suppressed at 10 weeks (21 weeks of age) after whole-body irradiation (2Gy; 65%, 4Gy; 44% ($p < 0.05$) in Table 1). Following the acute phase of immunosuppression, however, the tumor reduction rate had returned to a level comparable to those of the controls at 22 weeks after whole-body irradiation. After 54 weeks (65 weeks of age), the irradiated mice showed decreased tumor reduction rates with age, with values even lower than those of non-irradiated age-matched control mice; however, the difference was not statistically significant at the 5% level except for mice 65 weeks of age in the 4 Gy irradiated group.

Table 1. Changes in tumor growth rate and tumor reduction rate within two weeks after local irradiation to tumor as a function of age in control and irradiated C3H/He female mice.

Age in weeks	Tumor Growth Rate			Tumor Reduction Rate		
	Control group	Irradiated Groups 2 Gy	4 Gy	Control group	Irradiated Groups 2 Gy	4 Gy
21	1.47 ± 0.08 (12)	1.49 ± 0.10 (6)	1.92 ± 0.26 (6)	0.76 ± 0.02 (12)	0.65 ± 0.12 (6)	0.44 ± 0.21* (6)
33	1.36 ± 0.13 (12)	1.64 ± 0.13 (12)	1.66 ± 0.09 (12)	0.80 ± 0.06 (12)	0.75 ± 0.06 (12)	0.74 ± 0.07 (12)
65	1.52 ± 0.09 (8)	1.84 ± 0.21 (9)	1.58 ± 0.11 (9)	0.61 ± 0.06 (12)	0.42 ± 0.09 (10)	0.21 ± 0.09* (10)
80	1.63 ± 0.11 (9)	1.60 ± 0.12 (9)	1.61 ± 0.14 (6)	0.42 ± 0.09 (9)	0.29 ± 0.06 (8)	0.17 ± 0.16 (9)
96	1.78 ± 0.20 (6)	1.81 ± 0.12 (6)	1.69 ± 0.13 (6)	0.25 ± 0.19 (6)	0.14 ± 0.20 (6)	0.06 ± 0.26 (6)
102	1.81 ± 0.20 (6)	N.D.	N.D.	0.03 ± 0.21 (6)	N.D.	N.D.

Results are expressed as mean values ± standard errors with the number of mice in each experiment given in brackets.
Significant differences between the means of the irradiated and control groups of mice of similar age (Student's t-test).
*$p < 0.05$
N.D. experiment not done.

In a subsequent *in vitro* experiment, essentially similar results were obtained when the anti-tumor cell-mediated immune activity was assessed for individual spleens from these mice (Fig. 5). The results indicated that the initial dose-dependent suppression of the growth inhibitory activity of spleen cells was followed by a subsequent recovery during the few months after whole-body irradiation. After 22 weeks (33 weeks of age), there was no significant difference in the inhibition activity of the spleen cells between irradiated and non-irradiated age-matched control mice, as shown in Fig. 5. The age-dependent decline of the growth inhibitory activity of spleen cells appeared from 80 weeks of age in the non-irradiated controls and the 2-Gy irradiated mice. In contrast to the controls, the growth inhibition of tumor cells by spleen cells from 4-Gy irradiated mice began to decrease at about 65 weeks of age, and was somewhat lower than that of the age-matched control mice, but there was no significant difference in the means of inhibition rate at all time points examined between the two groups.

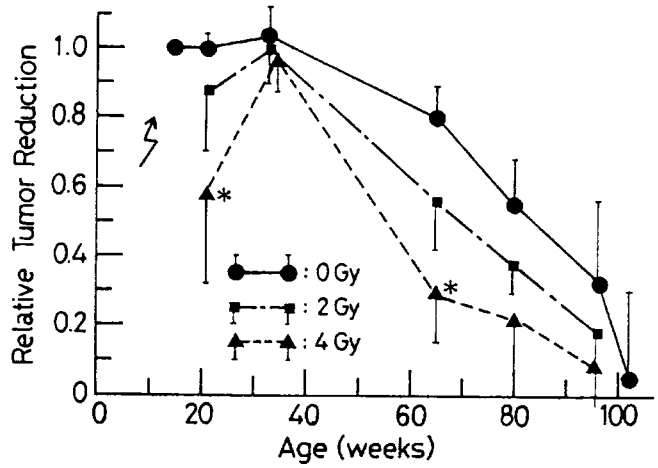

Fig. 4. Tumor reduction rate after local irradiation to tumor as a function of age for controls (●——●) and irradiated mice (■- - -■; 2 Gy, ▲- - -▲; 4 Gy). Average values of 6 to 12 mice with duplicate experiments and standard errors are shown.
* significant differences ($p < 0.05$).

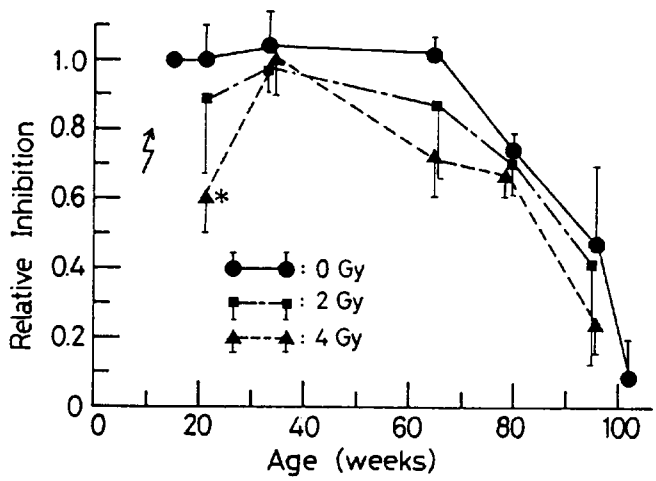

Fig. 5. Growth inhibitory activity of spleen cells after tumor irradiation as a function of age for controls (●——●) and irradiated mice (■- - -■; 2 Gy, ▲- - -▲; 4 Gy). Average values of 6 to 8 mice with duplicate experiments and standard errors are shown.

DISCUSSION

Inbred C3H/He female mice were exposed to whole-body irradiation of 2 and 4 Gy of 250 kVp X-rays at the age of 10—12 weeks. They were used for survival studies as well as analysis of age-related changes in immunological activity at varying intervals after whole-body irradiation.

There was a striking dose-dependent reduction of survival times of the irradiated mice. The median survival time of the controls was 98.6 weeks while the median life-span of the mice exposed to 2 and 4 Gy was shortened by 14.9 and 23.4 weeks, respectively (Fig. 3). This result is in agreement with the work of Storer[20], who reported that with radiation doses ranging from 100 to 1000R shortening of median survival was found to be a linear function of dose amounting to 45 days per 100R of X-ray dose. This result is also in general agreement with similar investigations by others[21,22].

The immunological activity of the host after tumor irradiation was investigated in *in vivo* and *in vitro* systems. The spleen cells from tumor irradiated mice inhibited growth of tumor in mice, and also inhibited colony formation and growth of tumor cells in a mixed lymphocyte tumor culture. In the suspension culture, when 5×10^4 tumor cells were incubated alone in 2 ml of complete culture medium, the cell population grew exponentially with a population doubling time of about 12.5 hr. There was no change in the growth rate when the tumor cells were incubated with normal spleen cells from C3H/He normal mice. However, spleen cells from mice with locally irradiated tumor markedly inhibited growth of tumor cells and spleen cells from mice with non-irradiated tumor slightly inhibited tumor cell growth. These spleen cells also inhibited colony formation in soft agar, and the inhibition rate reached maximum level at two weeks after tumor irradiation and continued for several more weeks[18].

The anti-tumor cell-mediated immune activity which is induced and/or enhanced by local irradiation to tumor gradually decreased with normal aging from 65 to 102 weeks of age in control mice, as judged by their capability of tumor reduction within two weeks after local irradiation to tumor. In contrast to the controls, the initial dose-dependent suppression of immunological activity was followed by a rapid recovery which was nearly complete at 22 weeks after whole-body irradiation. The age-dependent decline of immunological activity appeared earlier in the whole-body irradiated mice, and their tumor reduction rates were shown to be lower than those of the non-irradiated age-matched control mice, but the difference was not statistically significant at most time points examined, as shown in Fig. 4. Similar results were obtained when the growth inhibitory activity of spleen cells was assessed for individual spleens from these mice (Fig. 5).

Burnet[23] proposed that the age-dependent decline of immunologic functions is due primarily to a progressive exhaustion of a "quota" of cell divisions allowed for each stem cell in the immune system. Afterwards, this idea was explained by Sado[7] as follows: if each stem cell had a quota of cell divisions, a significant part of which had been used up during normal development of immune system, the residual quota of cell divisions allowed for each stem cell present at the time of radiation exposure may be considerably limited, resulting in insufficient recovery, or the regenerated immunological functions may decline earlier than in the control animals. Sado *et al.* reported[6,7,12] that the barrier-sustained specific-pathogen-free (SPF) C3Hf/HeMsNrs male mice exposed as young adults (10—11 weeks of age) to sublethal doses showed no effects of radiation on age-related changes in the immune response potential when assessed by primary antibody response of intact mice to SRBC, mitogen responsiveness of spleen cells *in vitro,* and cytotoxic T cell response of spleen cells *in vitro*. Therefore, they concluded that the aging of the immunological functions was not accelerated in these irradiated mice.

On the other hand, however, Peterson *et al.*[24] investigated the recovery of immune com-

petence following sublethal X-irradiation of young and old mice and found that most of the immunological parameters, e.g., response to SRBC, phytohemagglutinin, and bacterial lipopolysaccharide, showed delay in the time of recovery in old mice suggesting that age-related changes occur in the stem cells themselves and recovery of immunological activities in old mice is delayed partly because of the inability of their stem cells to rapidly generate immunocompetent progeny. Furthermore, Hayakawa *et al.*[11] reported that the long-term impairment of graft-vs-host reactivity after 600R of acute exposure is not due to defects intrinsic to the cells, but rather to extrinsic defects, such as failure of a regulatory factor to support the successful maturation and proliferation of cells.

Yuhas[10] showed very interesting data strongly indicating accelerated aging of the antibody response potential of BALB/C mice that had been exposed to whole-body irradiation of 5 Gy at a young age. Following the acute phase of immunosuppression, there was no detectable difference between irradiated and control responses until late in life. The senescent decline in immunological reactivity appeared earlier in the irradiated group suggesting that their stem cell reserves were reduced by the exposure. These findings regarding the time course of immune reaction following whole-body irradiation are generally consistent with those in our study presented here. In part, this discrepancy is probably related to the difference in the maintenance conditions of experimental mice. The experiments in Sado's reports were performed using SPF mice, maintained under barrier-sustained SPF conditions from birth to the time of assessment of immunological functions, while Yuhas and our studies were performed under conventional conditions. It is known that infection with viruses may reduce the immune response potential of mice[25]. Moreover, Anderson *et al.*[5] noted that the relationship of the immune response to tumor growth is analogous to the balance between immunity and infection, and radiation depresses immunity to permit greater spread of infection.

In conclusion, studies on the long-term effect of whole-body irradiation on immunological function in mice show that the specific anti-tumor cell-mediated immune response, which is induced and/or enhanced by local irradiation to tumor, was reduced in a dose-dependent manner when assessed 10 weeks after whole-body irradiation, but it recovered to the near-complete level of the non-irradiated age-matched controls within a few months, then gradually decreased with normal aging. From the point of view of late effects, these results suggest that the age-dependent decline of this immunologic activity appears earlier as a result of whole-body X-irradiation, and that the earlier appearance in the irradiated mice is probably related to accelerated aging as induced by irradiation. This phenomenon constitutes a newly found late effect of radiation on immune system, which has a direct bearing on tumor control.

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