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Suppression of Thymic Lymphoma Induction by Life-Long Low-Dose-Rate Irradiation Accompanied by Immune Activation in C57BL/6 Mice

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The induction of thymic lymphomas by whole-body X irradiation with four doses of 1.8 Gy (total dose: 7.2 Gy) in C57BL/6 mice was suppressed from a high frequency (90%) to 63% by preirradiation with 0.075 Gy X rays given 6 h before each 1.8-Gy irradiation. This level was further suppressed to 43% by continuous whole-body irradiation with ^{137}Cs γ rays at a low dose rate of 1.2 mGy/h for 450 days, starting 35 days before the challenging irradiation. Continuous irradiation at 1.2 mGy/h resulting in a total dose of 7.2 Gy over 258 days yielded no thymic lymphomas, indicating that this low-dose-rate radiation does not induce these tumors. Further continuous irradiation up to 450 days (total dose: 12.6 Gy) produced no tumors. Continuously irradiated mice showed no loss of hair and a greater body weight than unirradiated controls. Immune activities of the mice, as measured by the numbers of CD4$^+$ T cells, CD40$^+$ B cells, and antibody-producing cells in the spleen after immunization with sheep red blood cells, were significantly increased by continuous 1.2-mGy/h irradiation alone. These results indicate the presence of an adaptive response in tumor induction, the involvement of radiation-induced immune activation in tumor suppression, and a large dose and dose-rate effectiveness factor (DDREF) for tumor induction with extremely low-dose-rate radiation.

INTRODUCTION

Thymic lymphomas in C57BL mice are induced most efficiently by four doses of 1.8 Gy of whole-body X radiation, providing a total dose of 7.2 Gy, according to the original protocol of Kaplan and Brown (*1, 2*). The thymic lymphomas produced by this regimen are monoclonal (*3*). In this study, we employed this system for examining the effect of low-dose and low-dose-rate radiation on tumor induction.

Low-dose radiation activates the immune system of mice (*4–12*), delays the formation of radiation-induced mouse leukemia in an adaptive manner (*13*), and suppresses spontaneous tumors in AKR mice (*14*) and in heterozygous *Trp53*-mutant mice (*15*). Recently, MRL-*lpr/lpr* mice with severe autoimmune diseases showed a dramatic prolongation of life span together with immune modification and suppression of total-body lymphadenopathy when they were irradiated continuously with γ rays at a low dose rate of 1.2 mGy/h for 5 weeks (*16*). Furthermore, low-dose radiation increases the level of cellular glutathione (*17*) and enhances DNA excision repair (*18*). Sensitivity to tumor induction with ionizing radiation is dependent on Trp53 function (*19*), which is inducible with low-dose radiation (*20*).

Based on the above findings on radiation-inducible activities, we expected that the induction of thymic lymphomas by high-dose radiation at a high dose rate would be suppressed by low-dose and low-dose-rate radiation and further that the adaptive response (*21*) first demonstrated in chromosome aberrations in cultured human lymphocytes (*22*) would occur for tumor induction in the whole body. The adaptive response for mouse survival has been demonstrated (*23*).

On the other hand, experiments with continuous irradiation at a low dose rate involve another important problem of the dose-rate effects in the action of ionizing radiation. The tumor induction efficiency of radiation is greatly influenced by dose rate even for the same total dose. Reduction of radiation-induced tumors at low dose rates of low-LET radiation has been observed in previous work (*24–27*). The currently adopted value for the dose and dose-rate effectiveness factor (DDREF) of low-LET radiation for radiation protection purposes is 2 (*28*). However, recent observations on radiation-induced mouse leukemia in the dose-rate range of 0.04–1189 mGy/min indicate that this value is in the range 20–45, although the DDREF for solid tumors was smaller (*29*). A high DDREF is also indicated from the wide variation of tumor induction efficiency as shown in a review of non-tumor-inducing doses of radiation by com-

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paring the values obtained after acute, protracted and chronic irradiations (*30*). Therefore, the factor 2 for DDREF is applicable only to a relatively high-dose-rate range. In the extremely low-dose-rate ranges near environmental levels, the DDREF value must be much higher.

For evaluating dose-rate effects in the extremely low-dose-rate range, experimental and human data on internal emitters are very useful. The dose-rate effect was studied extensively using β particles from tritium administered to B6C3F1 (C57BL/6 × C3H/He, F_1) mice in drinking water over a wide range of concentrations. These results showed that no thymic lymphomas were produced below a threshold of 0.9 mGy/day (*31–33*). Such a threshold-like dose-rate effect was also observed for tumor formation with external local β-particle irradiation on mouse skin (*34*). It is interesting to see how the high efficiency of a total dose of 7.2 Gy in induction of thymic lymphoma is affected by delivering the same total dose at a very low dose rate.

In this report, we will show the presence of an adaptive response in the induction of mouse thymic lymphoma and the suppression of tumor induction by continuous low-dose-rate irradiation, accompanied by immune activation. We will further show the absence of tumors in mice chronically irradiated at a very low dose rate.

MATERIALS AND METHODS

Animals

Female C57BL/6N Jcl mice (5 weeks old) were purchased from Clea Japan, Inc. (Tokyo) and were kept under specific-pathogen-free conditions. All the animals were maintained on a light schedule from 7:00 to 19:00 and were fed a standard mouse diet CE-2 (Clea Japan) with water allowed *ad libitum.* The study was reviewed by the Institutional Animal Care and Use Committee, and the mice were treated in accordance with governmental guidelines and the guidelines of the Central Research Institute of Electric Power Industry (CRIEPI). All mouse groups for tumor experiments except group B were prepared concurrently. The mice in all groups had nearly equal body weights in the same range within 1 SE at the start of the experiment.

Irradiation

For acute irradiation, X rays were generated by a 300 kV generator (Model MBR-320R, Hitachi Medical Co., Tokyo) at 10 mA with filters of 1.0 mm aluminum and 0.2 mm copper and at a dose rate of 2.0 Gy/min at the mouse holder position as measured by a built-in ionization chamber. Continuous total-body irradiations with low-dose-rate γ rays were carried out in a clean irradiation room equipped with a 370 GBq ^{137}Cs γ-ray source (Chiyoda Technol Co., Tokyo) at the CRIEPI long-term low-dose-rate irradiation facility. Mouse cages were placed on shelves located 5 m from the source. The mice were irradiated continuously except for 1 h in the morning on weekdays from 5 weeks of age, starting on day 0 (Fig. 1). The dose rate was 1.2 mGy/h as determined from dosimetry with an ionization chamber. The tissue dose rate measured with a glass dosimeter that was embedded in a mouse's abdomen under the same irradiation conditions in other series of our experiments was 0.95 mGy/h (*35*). The design of the irradiation facility and the details of dosimetry with the ionization chamber and glass dosimeter have been described elsewhere (*35*).

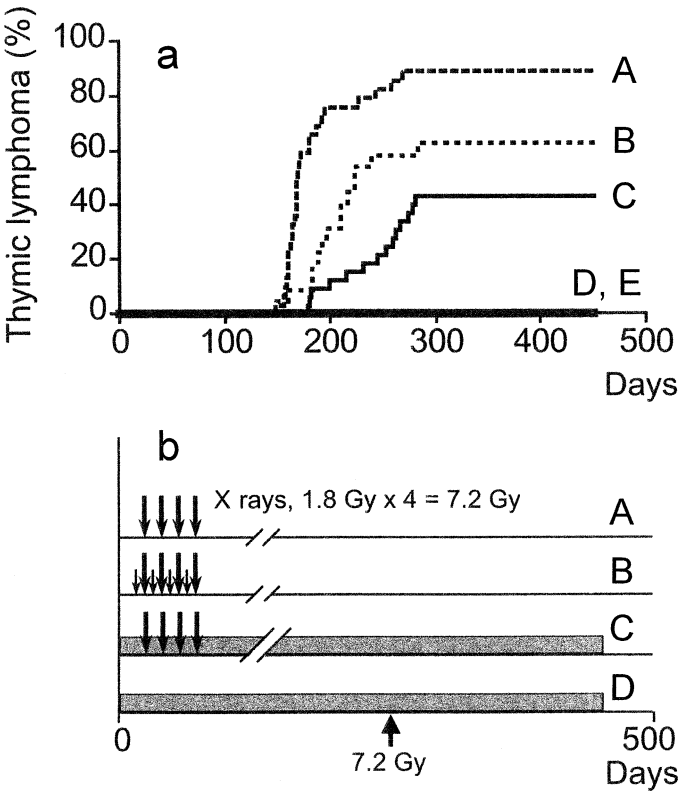

FIG. 1. Suppression of thymic lymphoma induction by acute low-dose or continuous low-dose-rate irradiation in C57BL/6 mice. Panel a: Cumulative incidence of thymic lymphomas plotted as a function of time. Panel b: Regimen of irradiation. (A) An acute dose of 1.8 Gy X rays was given four times once, every 7 days. Total dose: 7.2 Gy. (B) The four doses of acute 1.8 Gy X irradiation were combined with preirradiation with a low dose of 0.075 Gy X rays given before each 1.8-Gy X irradiation (test of adaptive response). (C) The four doses of acute 1.8 Gy X radiation were combined with continuous ^{137}Cs γ irradiation at a low dose rate of 1.2 mGy/h. (D) Continuous 1.2 mGy/h γ irradiation only. (E) No irradiation. Time zero was set at 5 weeks of age, when the continuous irradiation started. The upward arrow indicates the point at which the total cumulative dose of the continuous low-dose-rate irradiation reached 7.2 Gy. Difference in final tumor incidence: $P < 0.01$ for A − B, A − C and B − C; $P < 0.0001$ for A − D, B − D and C − D.

Regimens of Irradiation

Regimens for acute, continuous and combined irradiations of mice are shown in Fig. 1b. Numbers of mice in each group were 30 (group A), 22 (group B), 32 (group C), 20 (group D) and 20 (group E). All mice were taken into the laboratory and put in cages, five to six mice per cage at the age of 5 weeks (day 0). Group A mice were given four acute doses of X radiation with 1.8 Gy weekly beginning on day 35. Group B mice were given acute low-dose X irradiation with 0.075 Gy 6 h before every 1.8-Gy X irradiation. Group C mice were given continuous γ irradiation at 1.2 mGy/h from day 0 throughout life and in addition four doses of acute X irradiation with 1.8 Gy weekly beginning on day 35. Group D mice were given only continuous 1.2-mGy/h γ radiation throughout life. Group E mice were sham-irradiated in the irradiation chamber. Tumor experiments for the all groups started concurrently, except for group B, which started 1 year earlier with positive and negative controls.

Tumors

When tumors appeared at the cervix and enlarged, the mice were killed humanely and autopsied. At the moribund stage, mice showed irregular

breathing and unhealthy hair. Specimens of organs and tissues were fixed in 10% formalin/PBS solution. After embedding in paraffin, 3-μm-thick sections were prepared, stained with hematoxylin and eosin, and examined histologically under a microscope. Thymic lymphomas were confirmed from a high density of lymphoma cells in the tumor mass. Cumulative thymic lymphoma incidence rates were calculated according to a modification of the Kaplan-Meier method (*36*).

Analysis of Immune Cell Status

Immunological analysis followed the methods as described previously (*37*). The C57BL/6 mice were immunized by intraperitoneal injection of 15% (v/v) sheep red blood cells (RBCs) in saline at a dose of 0.02 ml/g body weight. Only saline at the same dose was injected into control mice. Six mice were used for the measurements at each point. Four days after sheep RBC immunization, a single cell suspension was prepared from the spleen in RPMI 1640 medium supplemented with 10% FCS. Immunological changes were examined by analysis of cell populations that expressed various immune-specific surface molecules and also by analysis of the anti-sheep RBC antibody-producing cell populations. The T-cell (CD4) and B-cell (CD45R/B220, CD40) markers were analyzed by flow cytometry. $CD45R/B220^+$ $CD40^+$ cells, i.e. $CD40^+$ B cells, are the representative cell population in the activated immune status. The numbers of anti-sheep RBC antibody-producing cells were obtained after counting the plaque-forming cells (PFCs).

Antibodies for Flow Cytometry

For immunofluorescence studies, the following monoclonal antibodies (mAbs) from BD PharMingen (San Diego, CA) were used: purified 2.4G2 mAb (rat IgG2b, κ), which recognizes CD16/32 (Fcγ III/II receptor) for FcBlock; FITC-conjugated GK1.5 mAb (rat IgG2b, κ), CD4; FITC-conjugated RA3-6B2 mAb (rat IgG2a, κ), CD45R/B220; R-PE-conjugated 3/23 mAb (rat IgG2a, κ), CD40.

Flow Cytometry

The single cell suspensions of spleen cells were preincubated with unlabeled anti-Fcγ receptor mAbs for 10 min at 4°C to avoid non-specific Fc-mediated binding of the labeled antibodies. The cells were stained with FITC-conjugated mAbs and R-PE-conjugated mAbs simultaneously for 20 min at 4°C. After being washed, the stained samples were analyzed using the EPICS XL flow cytometry system (Beckman Coulter, Inc., Fullerton, CA).

Anti-Sheep RBC PFC Assay

To count the anti-sheep RBC antibody-producing cells, i.e., anti-sheep RBC PFCs, the mice were killed 4 days after the sheep RBC immunization when the titer of anti-sheep RBC antibody was at its peak. The single cell suspension was prepared from the spleen as described above. Incubation mixtures were prepared with 490 μl RPMI 1640 medium supplemented with 10% FCS, 10 μl spleen cell suspension, 50 μl of a 50% SRBC suspension, and 50 μl of a solution of complement to enhance the hemolytic reaction. After the suspension was mixed gently in a water bath at 37°C for 5 min, it was plated in Cunningham chambers that were sealed with paraffin. After incubation for 1 h at 37°C in a 95% air/5% CO_2 incubator, PFCs were counted in triplicate for each mouse.

Statistical Analysis

The significance of differences in the tumor incidence (percentage of mice with tumors) was evaluated by the logrank test. The percentages of immune cell populations and the PFC numbers were presented as means ± standard errors. The statistical significance of the results for immune cells was evaluated using the Student's *t* test.

RESULTS

Suppression of Thymic Lymphoma Induction

Figure 1a shows the time course of induction of thymic lymphoma, and Fig. 1b shows the regimen of irradiation. Four weekly doses of 1.8 Gy radiation (total dose: 7.2 Gy) yielded a high incidence (90%) of thymic lymphomas (Fig. 1ab-A). All of these tumors were histologically confirmed to be thymic lymphomas. Thus the results of the original work of Kaplan and Brown (*1*) were well reproduced.

Preirradiation of mice with an acute low dose of X rays, 0.075 Gy, given 6 h before each 1.8-Gy irradiation significantly suppressed the thymic lymphoma incidence to 63%, while positive control mice treated as group A gave 90% tumor incidence, indicating the presence of the adaptive response in tumor induction (Fig. 1ab-B). No further increase of tumors was found up to 1,026 days in group B. When the mice were exposed to low-dose-rate γ rays at a dose rate of 1.2 mGy/h, starting 35 days before the first 1.8-Gy challenging irradiation, the incidence of thymic lymphoma was further suppressed to 43% (Fig. 1ab-C). Tumors other than thymic lymphoma were not recognized. When the continuous 1.2 mGy/h irradiation was terminated at the end of the 1.8 Gy irradiations, tumor appearance was delayed for 20 days and a tendency toward suppression of tumor induction was observed, but the final incidence was not significantly different from that of mice given the 1.8-Gy irradiations alone (data not shown), indicating that full expression of tumor-suppressing activity requires continuation of irradiation.

Absence of Thymic Lymphomas in Continuously Low-Dose-Rate-Irradiated Mice

When our mice were exposed to continuous low-dose-rate γ radiation alone at 1.2 mGy/h, thymic lymphomas were not found during 450 days after the start of exposure (Fig. 1ab-D). All of the 20 mice survived without any tumor until day 450. It should be noted that during the continuous exposure at 1.2 mGy/h, the cumulative radiation dose on day 258 reached 7.2 Gy, the same total dose applied to obtain a high incidence of thymic lymphomas by the four doses of 1.8 Gy radiation, and on day 450 reached 12.6 Gy, which is beyond the lethal dose with acute irradiation. Furthermore, no tumor was found in the group D mice until 664 days (total dose: 18.6 Gy). This indicates that low-dose-rate irradiation is inefficient for tumor induction as well as for killing mice. All 20 unirradiated control mice survived until day 450 and had no thymic lymphomas (Fig. 1a-E). We also have 84 unirradiated and sham-irradiated control mice in other series of experiments that developed no thymic lymphomas during the observation period.

Enhancement of Immune Cell Activities

Sheep RBCs were injected into mice at each time step (0, 1, 3, 5, 9, 13 and 17 weeks after the start of low-dose-

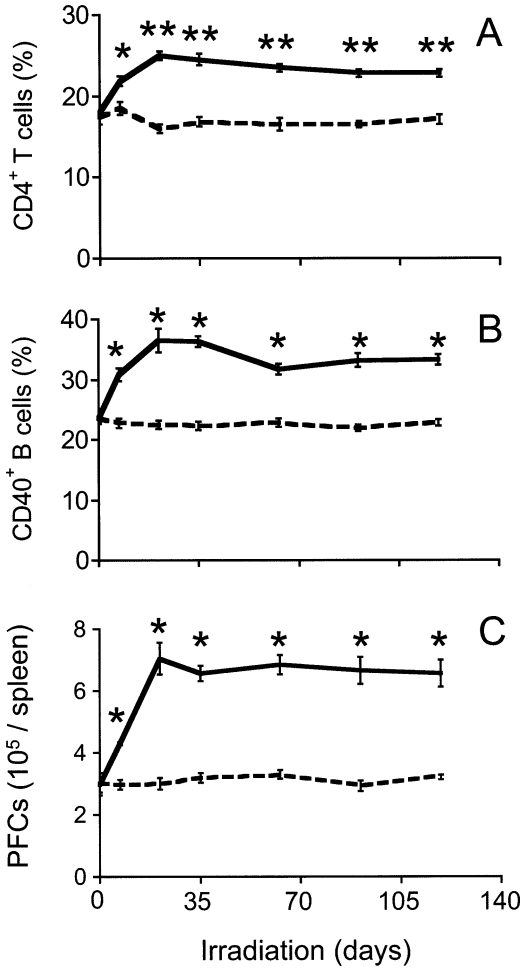

FIG. 2. Activation of immune cell populations in the spleens of C57BL/6 mice challenged intraperitoneally by sheep RBCs plotted as a function of time during continuous low-dose-rate γ irradiation from 5 weeks of age. $n = 6$ per point. The solid lines indicate the levels of the immune cells in the 1.2-mGy/h-irradiated mice, and the dotted lines indicate the levels of the immune cells in nonirradiated mice. Panel A: $CD4^+$ T cells ($^*P < 0.01$ and $^{**}P < 0.0001$ to the control). Panel B: $CD40^+$ B cells, i.e. activated B cells ($^*P < 0.0001$). Panel C: Plaque-forming cells, i.e. anti-sheep RBC antibody-producing cells ($^*P < 0.0001$).

rate irradiation) during continuous γ irradiation at 1.2 mGy/h to measure the immune responses of the mice. Populations of $CD4^+$ T cells and $CD40^+$ B cells (activated B cells) started to increase and reached a plateau 21 days after the start of irradiation. Thereafter, the elevated levels were maintained during the continuous irradiation (Fig. 2A and B). Splenic plaque-forming cells increased with time in response to sheep RBCs after the start of the continuous low-dose-rate γ irradiation (Fig. 2C). This level reached 2.3 times the level in the unirradiated mice. The pattern of the time course of this increase was very similar to that of $CD4^+$ T-cell and $CD40^+$ B-cell increases. These immunological activations shown in Fig. 2A, B and C were also observed in BALB/c and C3H/He mice. Furthermore, $CD4^+$ T-cell and $CD8^+$ T-cell populations increased 1.5-fold 2 days after acute X irradiation with 0.05–0.50 Gy at a high dose rate and returned to the levels seen in the unirradiated mice after 1 week (data not shown).

General Features of the Mice

Eighteen of the 20 unirradiated mice showed loss of hair and inflammation of the skin at the end of the experimental period. On the other hand, 17 of the 20 mice that were continuously irradiated at a low dose rate of 1.2 mGy/h for 450 days were hair-rich with shiny black coats (Fig. 3). The difference in the body weights between control and irradiated groups was first seen 2 months after the start of irradiation. On day 450, mean body weights were 33.7 ± 0.9 g for the nonirradiated mice ($n = 20$) and 40.5 ± 1.0 g for the continuously low-dose-rate-irradiated mice ($n = 20$). The difference between the two values was statistically significant ($P < 0.0001$).

DISCUSSION

In this study, we used the C57BL/6 mouse system for induction of thymic lymphomas with a high dose of radiation at a high dose rate and examined the modifying effects of acute low-dose and chronic low-dose-rate irradia-

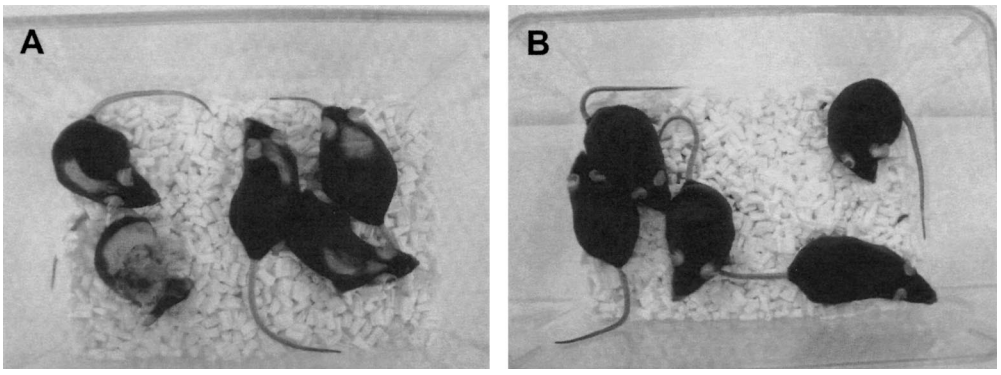

FIG. 3. Effect of continuous low-dose-rate γ irradiation on the appearance of C57BL/6 mice after 450 days. Panel A: Nonirradiated control mice. The normal loss of hair and inflammation of the skin are noted. Panel B: Mice irradiated at a low dose rate of 1.2 mGy/h from 5 weeks of age for 450 days. The mice were hair-rich with shiny black coats.

tion on the final tumor incidence. Although mouse thymic lymphoma is a specific type of tumor and may not represent tumors in general, hematopoietic tumors are most important in radiation carcinogenesis, and the thymic lymphoma system is useful for examining modifying factors in tumor induction. Such modifying factors include biological defense mechanisms inducible with low-dose-rate radiation. Currently, the mechanism for suppression of tumor induction is thought to involve DNA repair, elimination of injured cells by apoptosis (*38, 39*), and immune activation (*12, 16*).

The early experiments of Lorenz *et al.* with daily irradiation of LAF1 (C57L $\times$ A, F_1) mice at 0.11 r per day (8 h irradiation per day) found prolongation of the life span (*40*). However, tumors developed in the irradiated mice, in contrast to the absence of tumors in our C57BL/6 mice irradiated with 1.2 mGy/h. We interpret this difference to be the result of the difference in the dose rate used (0.11 mGy/h and 1.2 mGy/h; daily dose: 0.88 mGy and 27.9 mGy). There may exist an optimum dose rate for full expression of the tumor-suppressing effect of continuous irradiation above 1.2 mGy/h, as judged from the dose-rate response for immune activation (*16*).

The present study clearly showed the suppression of tumor formation by preirradiation with small doses, a result that was expected from the enhanced immune response that has been shown in past studies (*4–12, 16, 17*). The present results indicate the presence of an adaptive response in tumor induction in a whole-body system. Furthermore, continuous irradiation of mice with low-dose-rate radiation suppressed thymic lymphoma induction. The suppression of thymic lymphomas was found in parallel with the enhancement of immune activities. The immunological parameters used in the present study may not represent the whole tumor immunity, since the tumor immunity has not been fully elucidated and is considered to be more complex. However, we think that the parallel seen between tumor suppression and enhancement of immunological activities is a strong indication of the involvement of immune activation in tumor suppression by low-dose-rate radiation. It should be noted that low-dose-rate irradiation that ceased soon after termination of the challenging irradiation did not suppress the final incidence of thymic lymphoma, although a delay in tumor formation was observed. It is thought that preirradiation is insufficient for the full development of tumor immunity. The same situation is seen in the suppressing effect of caloric restriction on induction of mouse myelocytic lymphomas with radiation, where the suppressing effect is decreased by the termination of caloric restriction (*41*). Tumor suppression by immune activation may explain the inefficiency of low-dose-rate radiation for tumor induction. Furthermore, the mice continuously irradiated at a low dose rate of 1.2 mGy/h for 450 days were hair-rich with shiny black coats and weighed more than the nonirradiated mice. We think the activated immune system is the reason for their healthier conditions.

The absence of thymic lymphomas in the mice continuously irradiated at 1.2 mGy/h is another interesting point of this study. The total radiation dose, 7.2 Gy, which was reached on day 258 during the continuous irradiation, was equivalent to the total dose of four weekly doses of 1.8 Gy, which yielded a tumor incidence of 90%. The total dose given for the observation period of 450 days was even higher (12.6 Gy), and the dose reached 18.6 Gy on day 664 without tumor development (observation is being continued). In other series of our experiments examining the suppressive effect of low-dose-rate radiation on the chemical induction of subcutaneous tumors with 20-methylcholanthrene, we noted the absence of thymic lymphomas in mice surviving 330 days of 1.2 mGy/h irradiation for a total dose of 9.4 Gy (data not shown), again indicating the inefficiency of low-dose-rate irradiation for tumor induction even with the addition of the chemical carcinogen. Although a complete analysis of the dose-rate–response relationship for tumor induction requires extensive further experiments, a much higher DDREF value for a wide dose-rate range is now expected than had been thought previously.

The tumor-suppressive effect of low-dose-rate radiation may represent the hormetic response of biological systems to ionizing radiation (*5, 7, 42–44*). Immunological activation may be a part of this mechanism. Low-dose-rate radiation is thought to exert a qualitatively different response to ionizing radiation in biological systems, compared to the response to high-dose-rate radiation and to create a nonlinear dose response for tumor induction with low-dose-rate radiation.

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