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Prolongation of Life Span Associated with Immunological Modification by Chronic Low-Dose-Rate Irradiation in MRL-*lpr/lpr* Mice

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Chronic low-dose-rate γ irradiation at 0.35 or 1.2 mGy/h prolonged the life span of MRL-*lpr/lpr* mice carrying a deletion in the apoptosis-regulating *Fas* gene that markedly shortens life due to severe autoimmune disease. Immunological modifications as indicated by a significant increase of $CD8^+$ T cells and a significant decrease of $CD3^+$ $CD45R/B220^+$ as well as $CD45R/B220^+$ $CD40^+$ cells were found in parallel with amelioration of total-body lymphadenopathy, splenomegaly, proteinuria, and kidney and brain syndromes.

INTRODUCTION

At high doses, ionizing radiation is generally known to be harmful to living organisms. However, at low doses, radiation is no longer considered to be as harmful as once thought. Low-dose radiation can induce various effects on living organisms, including stimulation of growth rate (*1*), enhancement of survival after lethal high-dose irradiation (*2, 3*), prolongation of life span (*4–6*), activation of immune functions (*7–14*), increase of resistance to oxygen toxicity (*15*), improvement of mouse behavior (*16*), and prevention and cure of diseases (*17–21*). These stimulative effects of low-dose radiation have been called radiation hormesis (*22*). In the above studies, single or multiple acute doses of high-dose-rate radiation were used. Examination of the effects of chronic low-dose-rate irradiation is important from the standpoint of human health. Accordingly, in this study, we examined the effects of chronic low-dose-rate irradiation on the life span of mice with multiple severe diseases.

MRL-*lpr/lpr* mice were generated by cross-mating AKR/J, C57BL/6J, C3H/Di and LG/J mice (*23*); these mice have a deletion of the apoptosis-regulating *Fas* gene (*24*) that causes abnormal proliferation activity of lymphocytes, leading to severe total-body lymphadenopathy, splenomegaly and many autoimmune diseases, including glomerulonephritis, hepatitis, inflammation in blood vessels, joints, lung, skin, intestine and brain, and a very short life span (*25, 26*). The mice are animal models of systemic lupus erythematosus, rheumatoid arthritis and Sjögren's syndrome.

To cure MRL-*lpr/lpr* mice of these diseases using radiation, Moscovitch *et al.* applied local X irradiation, aiming at destruction of premalignant lymphocytes with a large total dose of 16 Gy given at a high dose rate (*27*). However, James *et al.* showed that the proliferative response of mouse spleen cells to a mitogen is increased by whole-body exposure to low-dose radiation at 0.01 Gy/day and 0.04 Gy/day at a high dose rate for 20 days. This response was found not only in C57BL/6-+/+ mice but also in C57BL/6-*lpr/lpr* mice, and it involves a mechanism different from that seen after a large dose (*9, 11*). However, its biological implications and molecular mechanism(s) remained unclear.

We report here that chronic whole-body irradiation of MRL-*lpr/lpr* mice at a low dose rate enhanced the immunological activities of lymphocytes as measured with various cell surface molecules and by a marked improvement in the symptoms of MRL-*lpr/lpr* mice together with a prolongation of their life span.

MATERIALS AND METHODS

Animals

Female MRL/MpJUmmCrj-*lpr/lpr* mice (5 weeks old) were purchased from Charles River Japan, Inc. (Yokohama) 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, Inc., Tokyo) 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).

Irradiation

Chronic 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-

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rate irradiation facility. Mouse cages were placed on shelves located 5 and 10 m from the source. The mice were irradiated continuously for 5 weeks at 7 to 12 weeks of age at 1.2 and 0.35 mGy/h each, as measured by an ionization chamber and glass dosimeter, except for 1 h in the morning on weekdays. The tissue dose rates measured with a glass dosimeter that was embedded in the mouse's abdomen were 0.95 and 0.30 mGy/h at each point. The design of the irradiation facility and the details of dosimetry with the ionization chamber and glass dosimeter have been described elsewhere (*28*). Shelves holding the cages containing the control mice were placed in the same room behind a wall that shielded them from the radiation.

Detection of Proteinuria

The clinical level of proteinuria was measured by the presence of protein in the urine. The urine was tested weekly using commercial test strips (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Histology

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 (H&E), and examined histologically under a microscope.

Analysis of Immune Cell Status

Single cell suspensions were prepared from the thymus and spleen in RPMI 1640 medium supplemented with 10% FCS. Immunological changes were examined in terms of cell surface molecules after analysis of cell populations. The cell surface functional molecules (CD3, CD4, CD8, CD45R/B220 and Fas) and activation-marker molecule CD40 were analyzed by flow cytometry.

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 (Fc-γ III/II receptor) for FcBlock; fluorescein isothiocyanate (FITC)-conjugated 17A2 mAb (rat IgG2b, κ), which recognizes CD3 molecular complex; FITC-conjugated GK1.5 mAb (rat IgG2b, κ), CD4; R-phycoerythrin (R-PE)-conjugated 53–6.7 mAb (rat IgG2a, κ), CD8a (Ly-2); FITC-conjugated and R-PE-conjugated RA3–6B2 mAbs (rat IgG2a, κ), CD45R/B220; R-PE-conjugated Jo2 mAb (hamster IgG, group 2, λ), Fas (CD95); R-PE-conjugated 3/23 mAb (rat IgG2a, κ), CD40.

Flow Cytometry

The single cell suspensions of thymus and spleen were preincubated with unlabeled anti-Fcγ receptor mAbs for 10 min at 4°C to avoid nonspecific 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 they were washed, the stained samples were analyzed by EPICS XL flow cytometry system (Beckman Coulter, Inc., Fullerton, CA).

Statistical Analysis

The Kaplan-Meier method was used to estimate the survival curves, and the significance of differences between the percentages of survival was evaluated by the logrank test. The significance of differences in the percentages of mice with lymphadenopathy or proteinuria was evaluated by the χ^2 test. The weights of spleen and inguinal lymph nodes and the percentages of cell populations are presented as means ± standard errors of the mean. The statistical significance of the differences was evaluated by the Student's *t* test.

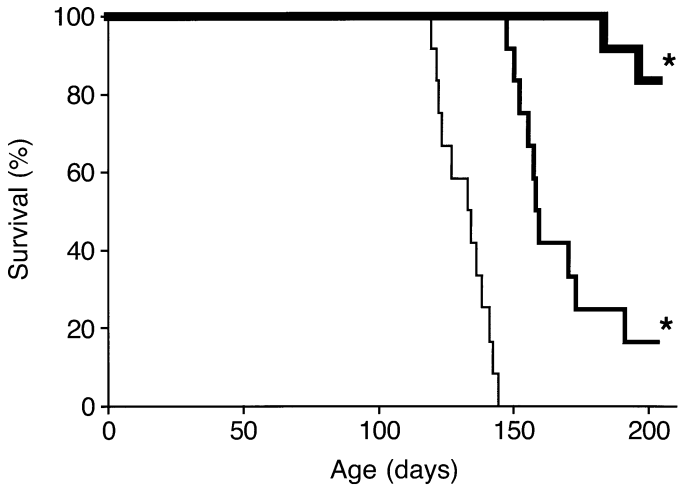

FIG. 1. Prolongation of life span in MRL-*lpr*/*lpr* mice with chronic low-dose-rate γ radiation. The animals were irradiated at three different dose rates for 5 weeks beginning at 7 weeks of age. The thinnest line represents nonirradiated control mice, the middle-width line represents mice irradiated at 0.35 mGy/h, and the widest line the mice irradiated at 1.2 mGy/h ($n = 12$ per group). The Kaplan-Meier method was used to estimate the survival curves, and the significance of differences between the percentages of survival was evaluated by the logrank test. $*P < 0.0001$ compared to controls.

RESULTS

Chronic Low-Dose-Rate γ Irradiation Prolonged Life Span of MRL-lpr/lpr Mice

Beginning at 7 weeks of age, MRL-*lpr/lpr* mice were kept in the γ-irradiation room and were irradiated at a dose rate of 0.35 or 1.2 mGy/h for 5 weeks. Thereafter they were maintained in the animal room without further exposure to radiation. Figure 1 shows the survival of the mice plotted as a function of age. It can be seen that the life span of MRL-*lpr/lpr* mice was markedly prolonged by chronic low-dose-rate irradiation and that this prolongation was dependent on the dose rate. Moreover, the irradiated mice appeared to have good coats of fur, to be active, and to breathe normally.

Chronic Low-Dose-Rate γ Irradiation Suppressed Lymphadenopathy, Proteinuria and Splenomegaly in MRL-lpr/lpr Mice

To obtain further insight into the effects of chronic low-dose-rate γ irradiation on the disease status of the mice, we observed lymphadenopathy, proteinuria and splenomegaly (Figs. 2 and 3), which are representative symptoms in MRL-*lpr/lpr* mice. These symptoms were markedly suppressed by chronic low-dose-rate irradiation at both 0.35 and 1.2 mGy/h. Furthermore, the amelioration of proteinuria was observed in the mice irradiated at low dose rates. We observed a notable decrease in the severity of proteinuria (data not shown) in addition to the decrease in the percentage of mice with proteinuria shown in Fig. 2B.

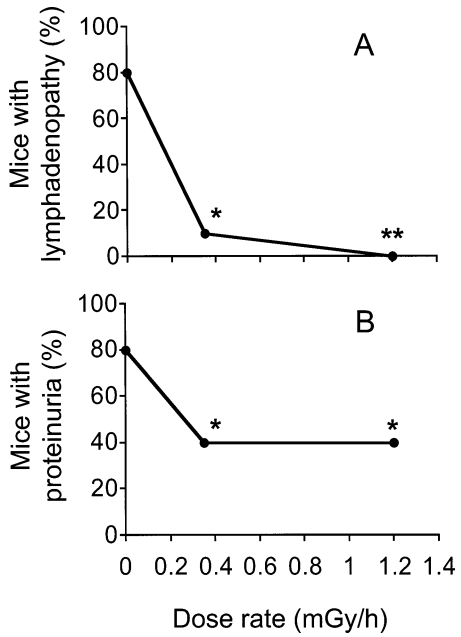

FIG. 2. Suppression of lymphadenopathy and proteinuria in MRL-*lpr*/*lpr* mice chronically γ-irradiated at low dose rates. The significance of differences in the percentages of mice with lymphadenopathy or proteinuria was evaluated by the χ^2 test. Panel A: Mice with palpable inguinal lymph nodes in each group at 12 weeks of age after 5 weeks of irradiation ($n = 10$ per group). $^*P < 0.01$, $^{**}P < 0.001$ compared to controls. Panel B: Mice with proteinuria over 30 mg/dl at 14 weeks of age 2 weeks after termination of the 5-week irradiation ($n = 10$ per group). $^*P = 0.068$ compared to controls.

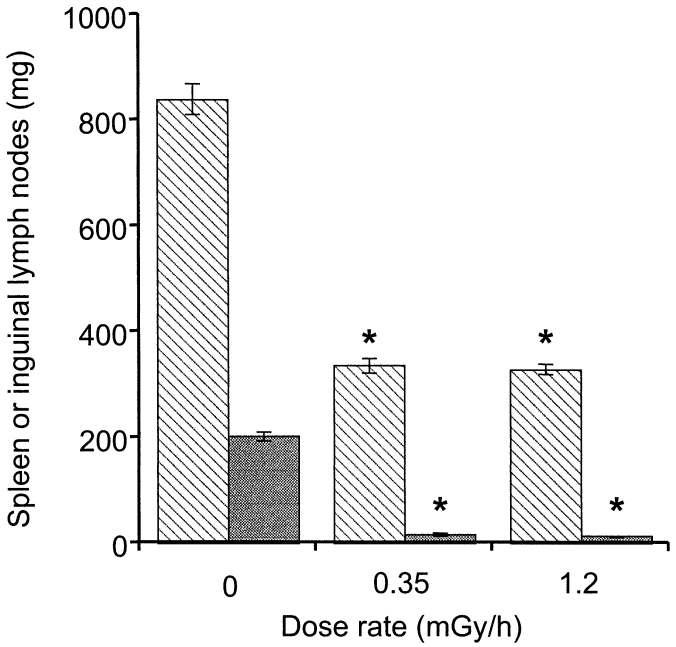

FIG. 3. The weights of the spleen (hatched bars) and the inguinal lymph nodes (solid bars) in the MRL-*lpr/lpr* mice at 12 weeks of age. Data for nonirradiated mice and mice γ-irradiated at low dose rates for 5 weeks are shown. The weights of the spleens and the inguinal lymph nodes are presented as means ± standard errors of the mean (SEM). Error bars represent the SEM. The statistical significance of the results was evaluated by the Student's *t* test ($n = 10$ per group). $^*P < 0.0001$ compared to controls.

Chronic Low-Dose-Rate γ Irradiation Improved Disease-Specific Damage to the Kidney and the Brain in MRL-lpr/lpr Mice

In parallel with the life-span study, we observed the effects of chronic low-dose-rate γ irradiation on the disease-specific damage to the kidney and the brain. In the chronically γ-irradiated mice, significant improvement of severe histological damage to the kidney and the brain, i.e. glomerulonephlitis in the kidney and hemorrhage and inflammation in the brain, was observed (Fig. 4). Amelioration of kidney syndrome, i.e. glomerulonephlitis, in the irradiated mice was observed in parallel with suppression of proteinuria. Furthermore, in the irradiated mice, the histological features, including elasticity of the whole brain, seemed normal.

Chronic Low-Dose-Rate γ Irradiation Decreased CD3⁺ CD45R/B220⁺ Cells and CD45R/B220⁺ CD40⁺ Cells and Increased CD8⁺ T Cells in MRL-lpr/lpr Mice

$CD3^+$ $CD45R/B220^+$ cells attack their own organs and tissues (*29*), and $CD45R/B220^+$ $CD40^+$ cells are a representative cell population in autoimmune diseases (*30*). The percentages of these cell populations are very high in MRL-*lpr/lpr* mice, although $CD3^+$ $CD45R/B220^+$ cells are absent and the numbers of $CD45R/B220^+$ $CD40^+$ cells are low in other mouse strains without the *lpr* mutation. These high levels of the abnormal cells characteristic of autoimmune diseases were significantly decreased in the low-dose-rate-irradiated *lpr* mice. Furthermore, the decreases in the cell populations were dependent on the dose rate (Fig. 5A and B).

In contrast, low-dose-rate radiation significantly increased the numbers of $CD8^+$ T cells, which are important cells for immune activity (Fig. 5C). Also noted was a tendency for an increase in the percentages of $CD4^+$ $CD8^+$ T cells in the thymus and $CD4^+$ T cells in the spleen after long-term irradiation at a low dose rate (data not shown), indicating improvement in immune status. Furthermore, no changes in the total cell numbers in the bone marrow and no injury in the intestine were observed (data not shown), indicating the absence of radiation damage.

DISCUSSION

In this study, we examined the effects of chronic low-dose-rate irradiation on MRL-*lpr/lpr* mice, which suffer from severe autoimmune disease and have a very short life span, and obtained the following results. First, chronic γ irradiation at low dose rates significantly prolonged the life span of the *lpr* mice. Second, the radiation suppressed total-body lymphadenopathy, splenomegaly and proteinuria, which are symptomatic of autoimmune injury. Third, kidney and brain syndromes were reduced significantly by

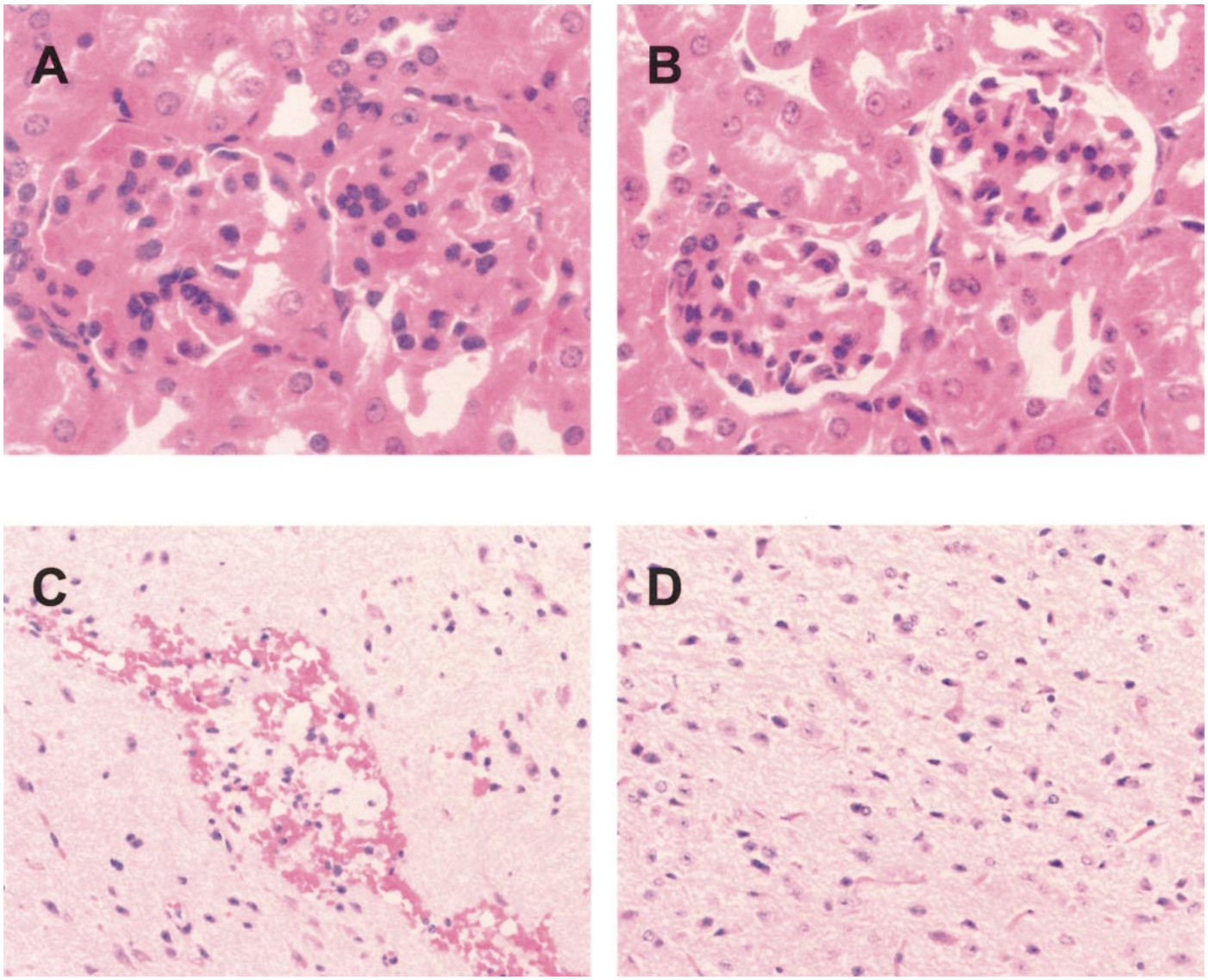

FIG. 4. Histological features of ameliorations of severe glomerulonephlitis in the kidney (H&E, original magnification 400×) and encephalitis in the brain (H&E, original magnification 200×) in MRL-*lpr/lpr* mice by chronic low-dose-rate γ irradiation. Panel A: Kidney of nonirradiated control mouse at 12 weeks of age. Severe glomerulonephlitis appeared. Panel B: Kidney of chronic low-dose-rate γ-irradiated mouse at 12 weeks of age after 5 weeks of irradiation at 1.2 mGy/h. Glomerulonephlitis did not appear, and normal morphological features were observed. Panel C: Brain of nonirradiated control mouse at 12 weeks of age. Severe hemorrhage and inflammation appeared as wide pink areas. Panel D: Brain of chronic low-dose-rate γ-irradiated mouse at 12 weeks of age after 5 weeks of irradiation at 1.2 mGy/h. Hemorrhage and inflammation did not appear, and normal histological features were observed.

chronic low-dose-rate irradiation. Fourth, the radiation significantly decreased the representative abnormal cell populations in the mice, i.e. $CD3^+$ $CD45R/B220^+$ cells and $CD45R/B220^+$ $CD40^+$ cells. The $CD3^+$ $CD45R/B220^+$ cells are autoreactive to their own organs and tissues throughout the body. The $CD45R/B220^+$ $CD40^+$ cells are the major cell population in the progression of autoimmune disease. Last, chronic γ irradiation at low dose rates significantly increased the numbers of $CD8^+$ T cells, which are considered to be most effective in the biological defense system.

It is our belief that the immunological modifications in the irradiated mice play important roles in disease suppression, because exposure to low-dose-rate radiation may stimulate the homeostatic system in *lpr* mice. Low-dose-rate irradiation is thought to activate the immune system by increasing immune-active cells and decreasing abnormal cells, although the details of this mechanism, including soluble mediators and signal transduction molecules, remain unclear.

Significantly increased numbers of $CD8^+$ T cells may attack abnormal cells effectively and therefore suppress the disease-specific abnormal cell populations, $CD3^+$ $CD45R/B220^+$ cells and $CD45R/B220^+$ $CD40^+$ cells, in low-dose-rate-irradiated *lpr* mice. The $CD45R/B220^+$ $CD40^+$ cells decreased to the levels seen in mice without the *lpr* mutation, although $CD3^+$ $CD45R/B220^+$ cells still existed in the

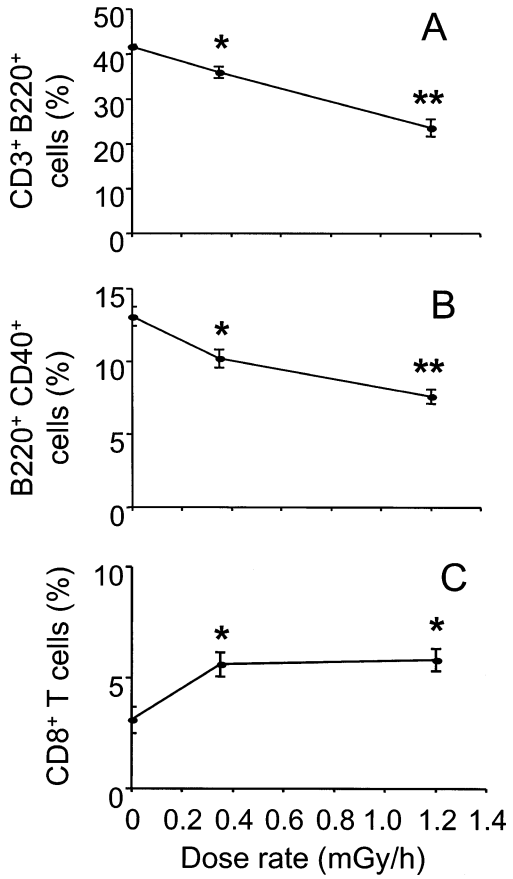

FIG. 5. Changes in immune cell populations in MRL-*lpr*/*lpr* mice chronically γ-irradiated at low dose rates at 12 weeks of age after 5 weeks of irradiation. The percentages of cell populations are presented as means ± standard errors of the mean (SEM). Error bars represent the SEM. The statistical significance of the results was evaluated by the Student's *t* test. Panel A: Percentage of spleen $CD3^+$ $CD45R/B220^+$ cells ($n = 10$ per group). $*P < 0.01$, $**P < 0.0001$ compared to controls. Panel B: Percentage of spleen $CD45R/B220^+$ $CD40^+$ cells ($n = 10$ per group). $*P < 0.01$, $**P < 0.0001$ compared to controls. Panel C: Percentage of spleen $CD8^+$ T cells ($n = 10$ per group). $*P < 0.01$ compared to controls.

irradiated *lpr* mice. An optimum dose rate for the decrease in abnormal cells may exist beyond the dose-rate range employed in this study. However, we believe that these decreases explain at least a part of the amelioration of severe whole-body autoimmune diseases, although other factors may also be involved.

The *lpr* mice spontaneously develop widespread inflammation in the cerebral vessels, meninges and choroid plexus. These mice are the first animal model for the human connective tissue diseases that damage the central nervous system (*25, 26*), and they provide a new model for studying this important clinical problem. In the present study, chronic low-dose-rate irradiation also significantly suppressed cerebral inflammation and hemorrhage in the *lpr* mice.

The 5-week low-dose-rate irradiation prolonged the life span of *lpr* mice after cessation of irradiation in the present study, indicating the persistence of the effect, although we have no data on the persistence of a corresponding effect on the immunological factors.

Apoptosis plays an important role in the immune system. Pathways to apoptosis through non-Fas-mediated mechanisms may exist in the low-dose-rate-irradiated *lpr* mice. In this study, there was no significant difference between the nonirradiated controls and the low-dose-rate γ-irradiated animals in terms of the expression of phosphatidylserine on the thymocytes and the percentages of thymocytes stained by propidium iodide, trypan blue and erythrosin B. Furthermore, expression of the apoptosis-regulating molecule Fas on the thymocytes and splenocytes was not observed in the chronically irradiated mice or the nonirradiated control mice (data not shown). We do not have data on the soluble mediators and signal transduction molecules that might mediate non-Fas-mediated pathways of apoptosis.

In previous studies using low-dose or high-dose radiation at a high dose rate or costimulatory molecule-targeted antibodies, activation of immune functions and amelioration of autoimmune symptoms in *lpr* mice were observed. James *et al.* found an increase in the numbers of $L3T4^+$ cells and lyt 2^+ cells in the spleens of C57BL/6-*lpr/lpr* mice after whole-body exposure to low doses of 0.04 Gy/day at a high dose rate for 2 days (*9, 11*). Recovery from glomerulonephlitis in the kidneys of MRL-*lpr/lpr* mice was reported after high-dose irradiation at a high dose rate (*27*). Sun *et al.* reported therapeutic effects of agonistic monoclonal antibodies against CD137 on symptoms in MRL-*lpr/lpr* mice (*31*). Mitchel *et al.* demonstrated that low-dose radiation modified the latency of radiation-induced myeloid leukemia in CBA/H mice (*32*) and increased the latency of spontaneous lymphomas and spinal osteosarcomas in cancer-prone, radiation-sensitive *Trp53* heterozygous mice (*33*). Tutt *et al.*, using mouse lymphomas, demonstrated that treatment with anti-CD40 monoclonal antibodies resulted in a rapid expansion of cytotoxic $CD8^+$ T cells, leading to long-term protection against tumor transplants (*34*). Furthermore, agonistic monoclonal antibodies against CD137 (4-1BB, Ly-63), a member of the tumor necrosis factor (Tnf) receptor superfamily that is a co-stimulatory molecule, have been shown to induce production of interferon γ (Ifng), rejection of tumor and allograft, and augmentation of $CD8^+$ T-cell responses (*35–39*). In the present study, similar immunological modifications were found with chronic low-dose-rate irradiation without apparent side effects.

In summary, we showed the amelioration of severe autoimmune diseases throughout the body, including kidney and brain syndromes, and prolongation of life span in parallel with the activation of immune system in *lpr* mice by chronic low-dose-rate irradiation without severe tissue damage. Thus low-dose-rate radiation may be useful in the treatment of autoimmune diseases.

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