

# Effect of Repeated Small-Dose $\gamma$ -Ray Irradiation on Atopic Dermatitis in NC/Nga Mice

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We previously showed that several small-dose 0.5 Gy whole-body  $\gamma$ -ray irradiation inhibits tumor growth in mice *via* elevation of the interferon (IFN)- $\gamma$ /interleukin 4 (IL-4) ratio concomitantly with a decrease in the percentage of B cells. Here, we examined whether repeated small-dose (0.5 Gy, 10 times)  $\gamma$ -ray irradiation influences atopic dermatitis in an NC/Nga mouse model. It was found that repeated  $\gamma$ -ray irradiation increased total IgE in comparison with the disease-control group. Levels of IL-4 and IL-5 were increased versus the disease-control group, while IFN- $\gamma$  was slightly decreased, resulting in a further decrease of the IFN- $\gamma$ /IL-4 ratio compared with the disease-control group. These results indicate that repeated small-dose  $\gamma$ -ray irradiation may exacerbate atopic dermatitis. This may be because the irradiation induces not helper T lymphocyte 1 (Th1), but Th2 polarization in this atopic mouse model, *i.e.*, the effects of small-dose irradiation may be different in conditions involving immune hypersensitivity and impaired immunity.

**Key words** —  $\gamma$ -ray, atopic dermatitis, NC/Nga mice

## INTRODUCTION

It is now well known that the response of the immune system to ionizing radiation depends on the dose and the dose-rate, and low-dose whole-body irradiation enhances the host immune response, rather than having a direct cell-killing action.<sup>1)</sup> In recent years, low-dose irradiation has been examined for the pre-clinical treatment of some chronic diseases, such as chronic lymphocytic leukemia and low-grade non-Hodgkin's lymphoma, and long-term remissions have been obtained.<sup>2)</sup>

Atopic dermatitis (AD) is a common pruritic disease that occurs primarily in infancy and childhood. The increase in the number of patients with atopic dermatitis has led to a requirement for new means to prevent the onset and to treat the disease. As Hanifin<sup>3)</sup> noted, AD is a chronically relapsing inflammatory skin disease with altered immune responses. It is associated with elevated Th activation, hyperstimulation of Langerhans cells,<sup>4)</sup> and a series

of immunological disturbances, such as overexpression of interleukin 3 (IL-3), IL-4, IL-5, IL-10, and granulocyte-macrophage colony stimulating factors (CSF) in the affected skin,<sup>5,6)</sup> decreased production of interferon (IFN)- $\gamma$  by T cells,<sup>7–9)</sup> and IgE hyperproduction of B cells.<sup>10,11)</sup> The defect of IL-12-induced IFN- $\gamma$  production may be a consequence of the hyperproduction of IL-4 and IL-10.<sup>12)</sup> These changes contribute to the up-regulation of humoral responses and the down-regulation of helper T lymphocyte 1 (Th1) responses.

To further investigate the pathophysiology and treatment of human AD, an animal model is required, and NC/Nga mice are suitable for this purpose. These mice were established as an inbred strain by Kondo in 1957 based on Japanese fancy mice (Nishiki-Nezumi), and various biological characteristics been reported, including high susceptibility to X-irradiation, and high susceptibility to ovalbumin (OVA)-induced anaphylactic shock.<sup>13,14)</sup> Inbred NC/Nga mice were confirmed to be available as an animal model for human AD:<sup>15)</sup> all the mice spontaneously developed AD-like skin lesions with a marked elevation in plasma levels of total IgE when they were raised in air-uncontrolled conventional circumstances (conventional NC/Nga mice). On the other hand, NC/Nga mice grown in a specific pathogen-

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free environment (SPF NC/Nga mice) showed neither clinical signs nor IgE hyperproduction. Immunohistochemical examination of the skin lesions in conventional NC/Nga mice presented features typical of the affected skin observed in patients with AD, such as an increased number of mast cells and eosinophils with marked degranulation, infiltration of numerous CD4<sup>+</sup> T cells and macrophages, and some infiltration of CD8<sup>+</sup> T cells. The activated mast cells and CD4<sup>+</sup> T cells in the skin lesions were positive for IL-4 and IL-5, but not for IFN- $\gamma$ .<sup>16)</sup> However, under similar conditions, BALB/c mice do not develop this form of dermatitis, indicating that strain-dependent genetic factors are involved.

Our previous studies have shown that several small-dose 0.5 Gy whole-body  $\gamma$ -ray irradiation inhibits tumor growth in mice.<sup>17,18)</sup> These anti-tumor effects were mediated by the radiation-induced elevation of the IFN- $\gamma$ /IL-4 (Th1/Th2) ratio concomitantly with a fall in the percentage of B cells. Here, the effect of repeated small-dose (0.5 Gy) whole-body  $\gamma$ -ray irradiation on atopic dermatitis was examined in the NC/Nga mouse model.

## MATERIALS AND METHODS

**Animals** — Six-week-old male NC/Nga mice, weighing 16–18 g, were purchased from Sankyo Laboservice Co. (Tokyo, Japan). They were housed in plastic cages with sterilized wood chip bedding and bred in rooms kept at a temperature of  $23 \pm 2^\circ\text{C}$  and a relative humidity of  $55 \pm 10\%$  under a 12 hr light-dark cycle. They were allowed free access to tap water and experimental normal diet (CE-2, CLEA Co., Ltd., Tokyo, Japan). Mice were divided into a normal group, a disease-control group and an irradiation group. Each group was consisted of five to six mice. All the mice were bred to 12 weeks old. Animals were treated and/or handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Tokyo University of Science. All experimental procedures mentioned below were in accordance with institutional guidelines for the care and use of laboratory animals in research.

**Reagents** — Dermatophagoides farinae crude extract (lyophilized, Torii, Tokyo, Japan) was used as a mite antigen. The following antibodies were used in this study: anti-mouse IFN- $\gamma$  mAb, biotin-

conjugated anti-mouse IFN- $\gamma$  mAb, anti-mouse IL-4 mAb, biotin-conjugated anti-mouse IL-4 mAb, anti-mouse IL-5 mAb, and biotin-conjugated anti-mouse IL-5 mAb (eBioscience, San Diego, CA, U.S.A.: XMG1.2, R4-6A2, 11B11, BVD6-24G2, TRFK 5 and TRFK 4, respectively). Recombinant mouse IFN- $\gamma$ , IL-4 and IL-5 were purchased from eBioscience (14-8311-63, 14-8041-62 and 14-7052-81, respectively). mAbs labeled with FITC, PE and Cy-Chrome and directed against CD3, T cell receptor associated complex present on all mature T cells; CD4, protein on helper T cells; CD8, protein on cytotoxic T cells; CD19, protein on B cells; and Pan-NK, protein on NK cells, were purchased from Becton Dickinson Co. (San Jose, CA, U.S.A.). All reagents used in this study were of reagent grade.

**Induction of Atopic Dermatitis Model** — From the 7th to the 11th week of age, mice of the disease-control and irradiation groups were injected with 10  $\mu\text{l}$  of mite antigen into the subcutaneous part of both ears twice a week (total 10 times). The mice of the normal group received mock sensitization with vehicle.

**$\gamma$ -Ray Irradiation** — The mice of the irradiation group were irradiated with 0.5 Gy of  $\gamma$ -rays 2 days before each stimulation, twice a week from the 7th to the 11th week of age (total 10 times). Mice were placed in an apertured lucite cabinet and given whole-body irradiation with  $\gamma$ -rays from a <sup>137</sup>Cs source (Gammacell 40, Nordin International, Inc., Canada) at a dose of 0.5 Gy (0.98 Gy/min) each time without anesthesia.

**Assay of Total IgE** — According to the procedure described by Watanabe *et al.*,<sup>19)</sup> blood was collected from the retro-orbital plexus with glass capillary tubes after completion of the irradiation treatment, and the plasma was separated. To determine the time-dependent changes in total IgE, blood samples were collected before stimulation and immediately after every stimulation. Total IgE was measured with an ELISA kit (Bethyl Laboratories, Inc., Montgomery, Texas, U.S.A.). The assay sensitivity was 3.9–250 ng/ml, and the samples were appropriately diluted.

**Assay of Cytokine Production by Spleen Lymphocytes** — Levels of IL-4, IL-5 and IFN- $\gamma$  produced by spleen lymphocytes were assayed as described by Wang *et al.*<sup>20)</sup> Briefly, the spleen cells ( $6 \times 10^6$  cells/ml) were harvested from the mice of each group after the last stimulation, suspended in 10% FBS/RPMI 1640 medium and cultured at  $37^\circ\text{C}$  in a CO<sub>2</sub> incubator. The cells were cultivated for

three days without stimulation, and for a further four days with the mite stimulus. The levels of IL-4, IL-5 and IFN- $\gamma$  in the culture supernatant were measured with ELISA kits (Pharmingen Co., San Diego, CA, U.S.A.). The assay sensitivities were 15–2000 pg/ml (IL-4 system), 8–1000 ng/ml (IL-5 system) and 78–5000 pg/ml (IFN- $\gamma$  system).

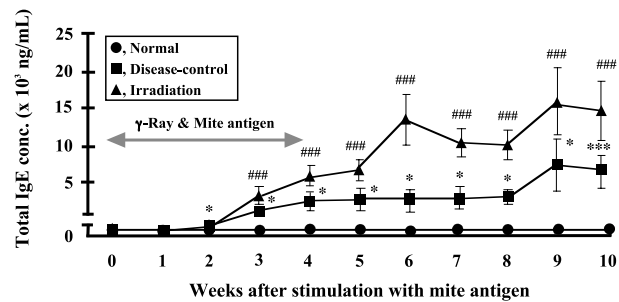
**Flow-Cytometric Analysis of Subpopulations of Spleen Lymphocytes** — Spleen lymphocytes were stained with PE-labeled anti-CD3, Cy-chrome-labeled anti-CD4, FITC-labeled anti-CD8, FITC-labeled anti-CD19 and PE-labeled anti-PanNK. All these antibodies were purchased from Pharmingen Co. Data were acquired using a FACS-Calibur flow cytometer (Becton Dickinson) and analyzed with Cell Quest software (Becton Dickinson).

**Statistical Analysis** — Values for all measurements were expressed as means  $\pm$  S.D. Groups were compared by the use of the Levene test, 1-way analysis of variance (ANOVA) and Scheffe test for comparison between two groups. *p*-Values of less than 0.05 were considered significant.

## RESULTS

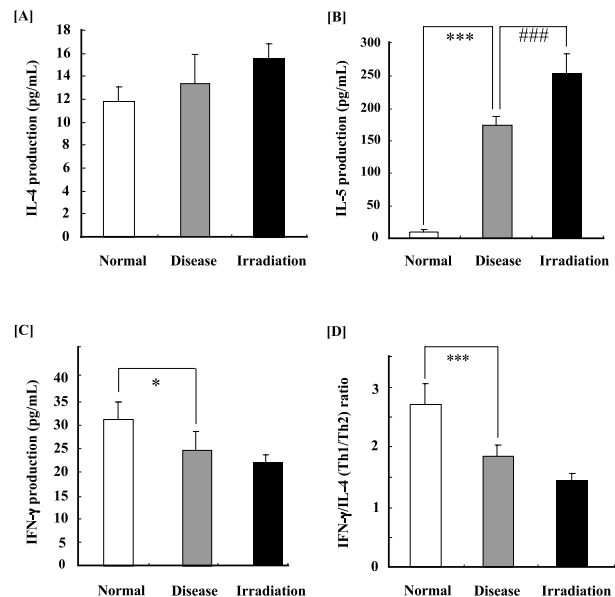
Time-dependent changes in the total IgE levels are illustrated in Fig. 1. The total IgE level in the atopic dermatitis disease-control group mice increased after the 3rd stimulation, reached a plateau at around the 5th stimulation, and thereafter remained significantly higher until the 10th stimulation. The irradiation group showed significantly higher values than the disease-control group at each point after the 3rd stimulation (Fig. 1).

The level of IL-4 in NC/Nga mice was slightly increased by the stimulation with mite antigen. Irradiation increased the level slightly further (Fig. 2[A]). The level of IL-5 in the disease-control group of NC/Nga mice was significantly increased to  $173.67 \pm 13.79$  (pg/ml), as compared with  $8.73 \pm 4.44$  (pg/ml) in the normal group. Irradiation further increased the level to  $252.67 \pm 30.75$  (pg/ml) (Fig. 2[B]). The level of IFN- $\gamma$  in the disease-control group of NC/Nga mice was significantly decreased to  $24.62 \pm 4.24$  (pg/ml), as compared with that in the normal group ( $31.46 \pm 3.51$  pg/ml). Irradiation further lowered the concentration somewhat (Fig. 2[C]). The ratio of IFN- $\gamma$ /IL-4 (Th1/Th2) in the disease-control group of NC/Nga mice was significantly reduced as compared with that in the non-stimulated normal group, and this was still the case



**Fig. 1.** Time-Dependent Changes of Total IgE Level in NC/Nga Mice

Total IgE in plasma was assayed after stimulation with mite antigen (60  $\mu$ g/ml) from the 7th to 11th week of age. The values represent means  $\pm$  S.D. of 5 animals. Significant differences from the normal group are indicated with \* ( $p < 0.05$ ), \*\*\* ( $p < 0.001$ ), and a significant difference from the disease-control group with ### ( $p < 0.001$ ).

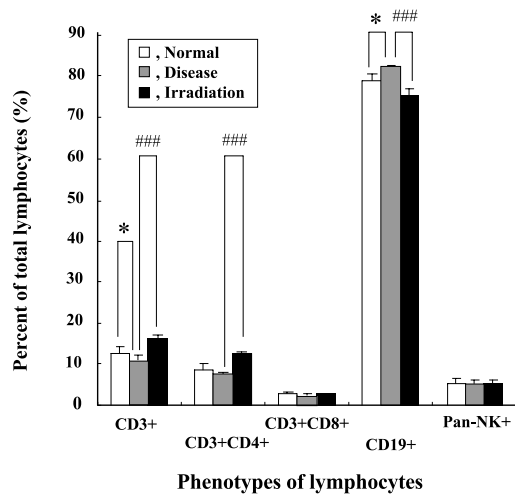


**Fig. 2.** Effect of Extended Small-Dose (0.5 Gy)  $\gamma$ -Ray Irradiation on the Level of IL-4, IL-5, IFN- $\gamma$ , and IFN- $\gamma$ /IL-4 (Th1/Th2) Ratio in NC/Nga Mice

Each value represents the mean  $\pm$  S.D. of 5 animals. Splenocytes ( $6 \times 10^6$  cells/ml) were harvested from the mice of each group 24 hr after the last stimulation, suspended in 10% FBS/RPMI 1640 medium and cultured at 37°C in a CO<sub>2</sub> incubator. The cells were cultivated for three days without stimulation, and for a further four days with the mite stimulus. Significant differences from the normal group are indicated with \* ( $p < 0.05$ ), \*\*\* ( $p < 0.001$ ), and those from the disease-control group with ### ( $p < 0.001$ ).

in the irradiated group (Fig. 2[D]).

The influence of irradiation on the subpopulations of spleen lymphocytes was examined in order to examine the effect on the Th1/Th2 balance from a different point of view. As shown in Fig. 3, a slightly lower percentage of CD3+, CD3+CD4+ and



**Fig. 3.** Effects of Extended Small-Dose (0.5 Gy)  $\gamma$ -Ray Irradiation on the Subpopulations of Splenic Lymphocytes in NC/Nga Mice

Splenic lymphocytes were harvested from the mice of each group 24 hr after the last stimulation. The average absolute numbers of total lymphocytes in normal, diseases-control, and irradiated group were  $2.51 \times 10^7$ ,  $3.37 \times 10^7$ , and  $1.68 \times 10^7$  cells/spleen, respectively. Immunophenotyping was carried out by using flow cytometry. Each value represents the mean  $\pm$  S.D. of 5 animals. Significant differences from the normal group are indicated with \* ( $p < 0.05$ ), and from the disease-control group with ### ( $p < 0.001$ ).

CD3+CD8+ T cells was observed in the disease-control mice, and irradiation slightly increased these percentages. An increased percentage of CD19+ cells was seen in the disease-control mice, and this increase was inhibited by irradiation. There was no significant change of Pan-NK cells among the 3 groups.

## DISCUSSION

Immune responses to protein antigens are strongly influenced by the nature of Th subsets. On the basis of their cytokine production profiles, Th cells are subdivided into two distinct populations, Th1 and Th2 cells. Th1 cells, through the production of IFN- $\gamma$ , evoke cell-dependent immunity and inhibit both the production of IL-4 by Th2 cells and Th2 cell proliferation *in vitro*.<sup>21,22</sup> In contrast, Th2 cells, through the production of IL-4, are responsible for the over-production of IgE. The cells can promote immunoglobulin class switching to IgG1 and IgE,<sup>23</sup> and also stimulate IgE gene recombination and transcription to IgE mRNA, thereby enhancing IgE production by B cells.<sup>24,25</sup> The balance between Th1- and Th2-dominant immunity (Th1/Th2

balance) has important effects on the development and status of various diseases. Predominance of Th2 and increased serum IgE level are seen in patients with atopic dermatitis.<sup>26,27</sup>

It is reported that an elevation of serum IgE levels was caused in NC/Nga mice by repeated epicutaneous application of mite antigen in conjunction with tape-stripping.<sup>28</sup> The dermatitis was accompanied with elevated serum IgE levels, accumulation of inflammatory cells including eosinophils in the lesions, and a skewing of the Th1/Th2 balance in favor of Th2. An increase in IL-4 mRNA expression and a decrease in IFN- $\gamma$  mRNA expression in cervical lymph nodes and ear lobes were also observed.

In our present study, we also observed an elevation of serum IgE levels accompanied with a Th2 predominance of the Th1/Th2 balance. The repeated small-dose (0.5 Gy)  $\gamma$ -ray irradiation increased the total IgE level above that of the disease-control group throughout the experiment, and induced a slight decrease of the IFN- $\gamma$ /IL-4 ratio. The Th2-dominant polarization of the Th1/Th2 balance is supported the fact that the levels of IL-4 and IL-5 in the irradiated mice were elevated above those in the disease-control mice.

Previously, it was found that several small-dose  $\gamma$ -ray irradiation (0.5 Gy, 4 times) elevated the IFN- $\gamma$ /IL-4 ratio and enhanced natural killer (NK) and cytotoxic T lymphocytes (CTL) activity, leading to delayed tumor growth, in Ehrlich solid-tumor-bearing mice. A lowering of the percentage of B cells and an increase in the percentage of CD4+ T cells were observed at 48 hr post-irradiation, and the ratio of IFN- $\gamma$ /IL-4 increased concomitantly with the decrease in the percentage of B cells.<sup>17,18</sup> Since atopic dermatitis is characterized by the production of large quantities of IgE antibodies by B cells and a decrease of IFN- $\gamma$ /IL-4 ratio, we anticipated that 0.5 Gy  $\gamma$ -ray irradiation might improve the status of this disease. Initially, we administered irradiation to the model mice just 2 days before each stimulation was repeated 5 times, but no effect was observed. When we changed the schedule of irradiation to a total 10 times, however, the levels of total IgE and IL-5 were increased to levels above those of the disease-control group, as shown in Figs. 1 and 2, and the IFN- $\gamma$ /IL-4 ratio was further decreased. It is noteworthy that irradiation inhibits Th1 polarization, but induces Th2 polarization in this disease model. This result would absolutely be supported by previous reports.<sup>29</sup> There might be three possible reasons for

these results. Firstly, the total dose of 5 Gy of radiation to the mice was quite high. Secondly, the sensitivity to irradiation may be strain-dependent. Shankar *et al.* indicated that the modification of immune response by radiation was different between BALB/c and C57BL/6 mice.<sup>30</sup> Thirdly, it would be supposed that irradiation might have an immunostimulatory effect on diseases involving impaired immunity, such as cancer and severe autoimmune disease (*e.g.*, MRL-*lpr/lpr* mice),<sup>31–35</sup> while exacerbating the response in immune hypersensitivity diseases, such as asthma.

In conclusion, repeated low-dose ionizing radiation may exacerbate the immune response in immune hypersensitivity diseases, such as asthma and atopic dermatitis. Further work is needed to establish the effects of low-dose whole-body irradiation in various chronic diseases.

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