J. Radiat. Res., 50, 573–577 (2009)

Induction of Late-onset Spontaneous Autoimmune Thyroiditis by a Single Low-dose Irradiation in Thyroiditis-prone Non-obese Diabetic-H2^{h4} Mice

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Autoimmune thyroiditis/Iodine/Low-dose irradiation/NOD-H2^{h4} mice.

The previous data regarding the effect of irradiation on thyroid autoimmunity are controversial. We have recently reported the exacerbation of autoimmune thyroiditis by a single low dose (0.5 Gy) of whole body irradiation in thyroiditis-prone non-obese diabetic (NOD)-H2^{h4} mice treated with iodine for 8 weeks. However, it is uncertain in that report whether the results obtained by the provision of iodine in a relatively short period of time (8 weeks) accurately reflects the long-term consequences of low-dose irradiation on thyroid autoimmunity. Therefore, we repeated these experiments with mice that were monitored after irradiation without iodine treatment for up to 15 months. We found that a single low-dose (0.5 Gy) irradiation increased the incidence and severity of thyroiditis and the incidence and titers of anti-thyroglobulin autoantibodies at 15 months of age. The numbers of splenocytes and percentages of various lymphocyte subsets were not affected by irradiation. Thus, we conclude that low-dose irradiation also exacerbates late-onset spontaneous thyroiditis in NOD-H2^{h4} mice; one plausible explanation for this may be the acceleration of immunological aging by irradiation.

INTRODUCTION

It is well known that irradiation exhibits various effects on the immune system. Although it is clear that high-dose irradiation suppresses immune responses by killing immune cells, the effects of low-dose irradiation on the immune system are controversial. For example, numerous *in vitro* studies and some *in vivo* experiments, particularly those on tumor immunity, have reported its enhanced effects on immune responses, while other *in vivo* experiments, especially those on autoimmunity, have demonstrated immune suppression by low-dose irradiation.[1)]

Focusing on the thyroid gland, which we believe is one of the most vulnerable organs to irradiation insult, the data regarding the effect of low-dose irradiation on thyroid autoimmunity are also inconsistent. Some, but not all, of the studies conducted in Chernobyl, the Marshall Islands, and the Nevada test site[2–9)] have demonstrated significant correlations between irradiation and thyroid autoimmunity. More importantly, one survey[10)] of atomic bomb survivors in Nagasaki and Hiroshima has also revealed a significant relationship between irradiation and thyroid autoimmunity, although this relationship was not found in other studies.[11–13)] Thus, it is presently unclear whether late effects of low-dose irradiation on the immune system exist.

We have recently reported that a single low-dose (0.5 Gy) irradiation of the entire body exacerbated autoimmune thyroiditis in thyroiditis-prone non-obese diabetic (NOD)-H2^{h4} mice.[1)] In that study, however, the incidence and severity of thyroiditis were determined in mice fed with iodine in their drinking water for 8 weeks. Thus, there is a concern that these results obtained by the provision of iodine in a relatively short period of time (8 weeks) may not necessarily accurately reflect the long-term consequences of low-dose irradiation on thyroid autoimmunity. Therefore, we repeated these experiments with mice that were monitored after irradiation without the provision of iodine for up to 15 months. We found, as in our previous study,[1)] that a single low-dose (0.5 Gy) irradiation increased the incidence and severity of thyroiditis and the incidence and titers of anti-thyroglobulin (Tg) autoantibodies at 15 months of age, indicating enhancement of the development of late-onset spontaneous thyroiditis in NOD-H2^{h4} mice.

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doi:10.1269/jrr.09067

MATERIALS AND METHODS

Mice

NOD-H2^{h4} mice were obtained from Jackson Laboratory Inc. (Bar Harbor, ME, USA) and bred with a regular diet (iodine contents of ~0.21 mg/100 g chow; CE2, Clea Japan, Tokyo, Japan) in the animal facility at Nagasaki University. Both male and female mice were used for the current study. Animal care and all experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with the approval of the Institutional Animal Care and Use Committee. All of the mice were kept in a specific pathogen-free condition.

Evaluation of thyroiditis

Thyroid tissues were removed and fixed in 10% formalin in phosphate-buffered saline. Tissues were then embedded in paraffin and 5-μm-thick sections were prepared and stained with hematoxylin-eosin. The severity of thyroiditis was expressed as one of five thyroiditis scores from 0 to 4, determined by the extent of lymphocyte infiltration as previously described.[1)]

Enzyme-linked immunosorbent assay for anti-thyroglobulin autoantibody measurements

Enzyme-linked immunosorbent assays for serum thyroglobulin (Tg) autoantibodies were performed as previously described.[1)] Briefly, assay wells were coated overnight with 100 μl Tg protein (10 μg/ml) and incubated with mouse sera (1:100 dilution). After incubation with horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich Corp., Tokyo, Japan), color was developed using orthophenylene diamine and H_2O_2 as substrate and the optical density was read at 492 nm (OD_{492}).

Thyroxine measurements

Serum-free thyroxine (T_4) concentrations were measured with a radioimmunoassay kit (DPC free T_4 kit; Diagnostic Products, Los Angeles, CA, USA).

Irradiation protocol

Anesthetized mice were exposed to a single 0.5- or 3-Gy dose of γ-irradiation with an EXS-300 γ-irradiator (200 kV; 15 mA; filter, 0.5 mm aluminum and 0.5 mm copper; 0.47 Gy/min; Toshiba, Tokyo, Japan).

Flow cytometry

The splenocytes were stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated anti-CD4 (H129.19; BD Biosciences, San Diego, CA, USA), anti-CD8 (53-6.7; BD Biosciences), anti-CD19 (1D3; eBioscience, San Diego, CA, USA), or anti-CD25 (7D4; eBioscience) antibodies according to the manufacturers' instructions and analyzed on a FACSCant II fluorescence activated cell sorter system (BD Biosciences).

Statistical analysis

Thyroiditis scores were analyzed by *t*-test and incidences of hyperthyroidism by the chi-square test. A p value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

To evaluate the long-term effect of radiation on thyroid autoimmunity in NOD-H2^{h4} mice, naïve mice and those irradiated with 0.5 or 3 Gy at 6 weeks of age were maintained on a regular diet for up to 15 months. Each group contained the same numbers (n = 12) of female and male mice. The data on thyroiditis, anti-Tg autoantibodies, and T_4 levels are summarized in Table 1, where the composite results from

Table 1. Thyroiditis, anti-thyroglobulin (Tg) autoantibodies, and free thyroxine (T_4) in control NOD-H2^{h4} mice and those exposed to irradiation.

	thyroiditis		anti-Tg autoantibodies		free T_4 (ng/dl)
	incidence	scores	incidence	titers	
9 months					
0 Gy	2/24 (8%)	0.06 ± 0.20*	4/24 (17%)	0.27 ± 0.17	n.d.**
0.5 Gy	7/24 (33)	0.60 ± 1.05	6/24 (25)	0.33 ± 0.24	n.d.
3 Gy	3/24 (13)	0.19 ± 0.65	4/24 (17)	0.26 ± 0.16	n.d.
15 months					
0 Gy	4/24 (17%)	0.39 ± 0.93	7/24 (29%)	0.30 ± 0.24	0.56 ± 0.16
0.5 Gy	**18/24 (75)** $^{\#}$	**2.03 ± 1.55** $^{\#}$	**16/24 (67)** $^{\#}$	**0.54 ± 0.39** $^{\$}$	0.47 ± 0.22
3 Gy	6/20 (30)	0.64 ± 1.46	7/20 (35)	0.30 ± 0.18	0.51 ± 0.30

*, mean ± S.D. (n = 20 – 24) ; **, not determined; $^{\#}$, $p < 0.01$ and $^{\$}$, $p < 0.05$ *vs.* the control (0 Gy-irradiated) group.

female and male mice are shown because female and male mice demonstrated essentially the same results. In 9-month-old mice, intrathyroidal lymphocyte infiltration was barely detectable in only 2 (8%) of 24 mice in the control group compared with 7 (33%, $p > 0.05$ by the chi-square test) of 24 mice in the 0.5-Gy irradiated group and 3 (12.5%, $p > 0.05$) of 24 mice in the 3-Gy irradiated group. There was also no significant difference in the degrees of thyroiditis among the three groups. Thus, thyroiditis scores were 0.06 ± 0.20 (mean ± S.D.) in the control group compared with 0.60 ± 1.05 ($p > 0.05$ by *t*-test) in the 0.05-Gy irradiated group and 0.19 ± 0.65 ($p > 0.05$) in the 3-Gy irradiated group. Likewise, the incidences and the titers of anti-Tg autoantibodies were insignificantly different among the three groups. Thus, the incidences were 17% (4/24) in the control group, 25% (6/24; $p > 0.05$) in the 0.5-Gy irradiated group, and 17% (4/24) in the 3-Gy irradiated group. The titers of anti-Tg autoantibodies were 0.27 ± 0.17 OD_{492} in the control group, 0.33 ± 0.24 in the 0.5-Gy irradiated group, and 0.26 ± 0.16 in the 3-Gy irradiated group.

In contrast, analyses of 15-month-old mice demonstrated significant exacerbation of thyroid autoimmunity in the 0.5-Gy irradiated group. In this set of experiments, 4 of 24 mice in the 3-Gy irradiated groups died between 10 and 14 months and were omitted from this study. In two of these mice, thymoma-like tumors were found upon necropsy. Thus, the incidence of thyroiditis remained low (4/24, 17%) in the control group, but increased to 75% (18/24; $p < 0.01$) in the 0.5-Gy irradiated group and 30% (6/20; $p > 0.05$) in the 3-Gy irradiated group. Degrees of thyroiditis were 0.39 ± 0.93 in the control group compared with 2.03 ± 1.55 ($p < 0.01$) in the 0.5-Gy irradiated group and 0.64 ± 1.46 ($p > 0.05$) in the 3-Gy irradiated group. The incidences of anti-Tg autoantibodies were 29% (7/24) in the control group, 67% (16/24; $p < 0.01$) in the 0.5-Gy irradiated group, and 35% (7/20) in the 3-Gy irradiated group, and their titers were 0.30 ± 0.24 OD_{492} in the control group, 0.54 ± 0.39 ($p < 0.05$) in the 0.5-Gy irradiated group, and 0.30 ± 0.18 in the 3-Gy irradiated group. Despite these differences, no difference was found in serum T_4 levels among the three groups.

Based on our previous study[1] demonstrating the exacerbation of iodine-induced autoimmune thyroiditis in NOD-$H2^{h4}$ mice by low-dose irradiation in a short time of period (8 weeks), the present study shows increases in the incidence and severity of thyroiditis and in the incidence and titers of anti-Tg autoantibodies in low-dose-irradiated 15-month-old mice. Our data indicate a causal relationship between low-dose irradiation and late-onset spontaneous autoimmune thyroiditis. These results are consistent with a previous report on atomic bomb survivors[10] and a recent study of Chernobyl residents,[6] both of which showed a significant relationship between radiation exposure and the prevalence of thyroid autoimmunity. However, the former study shows a bell-shaped, convexed dose-response curve with the maximum prevalence at 0.7 Sv,[10] which fits well with our present data; the latter study shows a linear dose-response up to 10 Gy.[6] There are also numerous reports that are inconsistent with our data as mentioned in the Introduction, including more recently published data on atomic bomb survivors.[13] The reasons for these discrepant data are unclear, but may include differences in cohort populations, diagnostic techniques, dose distribution of the cohort members, and the definition of thyroid disease. Our results are also not reconciled with previous reports showing suppression by low-dose irradiation of other autoimmune diseases in animal models.[1]

In an attempt to seek the mechanisms of this enhancing effect, the numbers of splenocytes and percentages of $CD4^+CD25^+$ regulatory T (Treg) cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells were evaluated at the same time points (Table 2). However, no significant differences were

Table 2. Numbers of splenocytes and percentages of $CD4^+CD25^+$ Treg cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells in splenocytes from control NOD-$H2^{h4}$ mice and those exposed to irradiation.

	splenocytes ($x10^7$)	$\frac{CD4^+CD25^+ T}{CD4^+ T}$ (%)	$CD4^+$ T (%)	$CD8^+$ T (%)	$CD19^+$ B (%)
9 months					
0 Gy	3.50 ± 0.72*	8.83 ± 0.73	50.11 ± 2.92**	14.62 ± 3.80	25.61 ± 3.83
0.5 Gy	3.70 ± 0.95	8.58 ± 2.45	45.93 ± 8.71	19.69 ± 2.97	21.46 ± 10.86
3 Gy	4.17 ± 0.85	8.06 ± 0.36	38.87 ± 0.31	17.27 ± 4.58	26.57 ± 5.79
15 months					
0 Gy	4.00 ± 0.10	5.78 ± 0.75	42.68 ± 4.34	17.22 ± 3.37	37.38 ± 2.91
0.5 Gy	4.41 ± 0.45	5.72 ± 0.79	38.63 ± 1.22	21.54 ± 6.47	33.56 ± 3.46
3 Gy	5.64 ± 1.77	6.46 ± 0.51	41.28 ± 2.61	14.57 ± 5.69	37.63 ± 13.88

*, mean ± S.D., n = 3; **, $p < 0.05$ *vs.* the other two groups.

observed in these immune cell subsets among the three groups. It should be noted here that $CD4^+CD25^+$ T cells may contain activated effector T cells because CD25 can also be expressed after T cell activation.[14] These data do not agree with previous reports. It has been demonstrated that chronic low-dose rate irradiation induced increases in $CD4^+$ T cells and $CD8^+$ T cells and a decrease in B cells.[15] A single dose of 0.2 Gy is also reported to increase the number and percentage of $CD8^+$ T cells.[16] Although the reasons for these discrepant data are unclear, we could not observe any significant alterations in immune cell subpopulations in the acute[1] and chronic phases (the present study) following radiation exposure.

Recent studies indicate that irradiation causes chronic low-grade inflammation in atomic bomb survivors more than 50 years after radiation exposure.[17–19] Because some of the markers used for evaluating inflammatory responses (for example, tumor necrosis factor α and immunoglobulins) are also elevated with aging, it is now thought that radiation exposure accelerates aging. Because a previous study reported an increased prevalence of anti-thyroid autoantibodies with age,[20] one plausible explanation for our results may be accelerated immunological aging by irradiation, although it is uncertain from our present study whether low-dose irradiation accelerates the onset of autoimmune thyroiditis or increases the incidence of the development of thyroiditis. Future studies are needed to clarify this issue.

Furthermore, one should be cautious in terms of applying our data to humans. Because the mice used in this study were thyroiditis-prone, our data may suggest that low-dose irradiation can manifest latent autoimmune thyroiditis, but not induce *de novo* disease. In this regard, it has been reported that individuals with higher titers of autoantibodies to thyroid peroxidase, another thyroid-specific autoantigen, had a higher risk of hypothyroidism compared with those with lower titers of the antibodies.[6]

In conclusion, we have shown using thyroiditis-prone NOD-$H2^{h4}$ mice that a single low-dose (0.5 Gy) irradiation at 6 weeks of age increased the incidence and severity of thyroiditis and the incidence and titers of anti-Tg autoantibodies in 15-month-old mice, indicating the enhancement by low-dose irradiation of the development of late-onset spontaneous thyroiditis.

ACKNOWLEDGMENTS

This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan through the Nagasaki University Global COE Program.

REFERENCES

1. Nagayama, Y., Kaminoda, K., Mizutori, Y., Saitoh, O. and Abiru, N. (2008) Exacerbation of autoimmune thyroiditis by a single dose of whole body irradiation in NOD-$H2^{h4}$ mice. Int. J. Radiat. Biol. **84**: 761–769.
2. Vermigilio, F., Castagna, M. G., Volnova, E., Lo Priesti, V. P., Vincenzo, P., Moleti, M., Violi, M. A., Artemisia, A. and Trimarch, F. (1995) Post-Chernobyl increased prevalence of humoral thyroid autoimmunity in children and adolescents from a moderately iodine-deficient area in Russia. Thyroid. **9**: 781–786.
3. Vykhovanets, E. V., Chernyshov, V. P., Slukvin, I. I., Antipkin, Y. G. Vasyuk, A. N., Klimenko, H. F. and Strauss, K. W. (1997) 131-I dose-dependent thyroid autoimmune disorders in children living around Chernobyl. Clin. Immunol. Immunopathol. **84**: 251–259.
4. Lomat, L., Galburt, G., Quastel, M. R., Polyakov, S., Okeanov, A. and Rozin, S. (1997) Incidence of childhood disease in Belarus associated with the Chernobyl accident. Environ. Health Perspect. **105**(Suppl. 6): 1529–1532.
5. Pacini, F., Vorontsova, T., Molinaro, E., Kuchinskaya, E., Agate, L., Shavrova, E., AstaChova, L., Chiovato, L. and Pinchera, A. (1998) Prevalence of thyroid autoantibodies in children and adolescents from Belarus exposed to the Chernobyl radioactive fallout. Lancet. **352**: 763–766.
6. Ostroumova, E., Brenner, A., Oliynnyk, V., McConnel, R., Robbins, J., Terekhova, G., Zablotska, L., Likhtarev, I., Bouville, A., Shpak, V., Markov, V., Masnyk, I., Ron, E., Tronko, M. and Hatch, M. (2009) Subclinical hypothyroidism after radioiodine exposure: Ukrainian-American cohort study of thyroid cancer and other thyroid diseases after the Chornobyl accident (1998–2000). Environ. Health Perspect. **117**: 745–750.
7. Kerbers, R. A., Till, J. E., Simon, S. L., Lyon, J. L., Thomas, D. C., Preston-Martin, S., Rallison, M. L., Lloyd, R. D. and Stevens, W. (1993) A cohort study of thyroid disease in relation to fallout from nuclear testing. J. Am. Med. Assoc. **270**: 2076–2082.
8. Cronkite, E. P., Bond, V. P. and Conard, R. A. (1995) Medical effects of exposure of human beings to fallout radiation from a thermonuclear explosion. Stem Cells. **13**(Suppl 1): 49–57.
9. Takahashi, T., Fujimori, K., Simon, S., Bechtner, G., Edwards, R. and Trott, K. (1999) Thyroid nodules, thyroid function and dietary iodine in the Marshall Islands. Int. J. Epidemiol. **28**: 742–749.
10. Nagataki, S., Shibata, Y., Inoue, S., Yokoyama, N., Izumi, M. and Shimaoka, K. (1994) Thyroid diseases among atomic bomb survivors in Nagasaki. J. Am. Med. Assoc. **272**: 364–370.
11. Morimoto, I., Yoshimoto, Y., Sato, K., Hamilton, H. B., Kawamoto, S., Izumi, M. and Nagataki, S. (1987) Serum TSH, thyroglobulin and thyroidal disorders in atomic bomb survivors exposed in youth: 30 year follow-up study. J. Nuc. Med. **28**: 1115–1122.
12. Yoshimoto, Y., Ezaki, H., Etoh, R., Hiraoka, T. and Akiba, S. (1995) Prevalence rate of thyroid diseases among autopsy cases of the atomic bomb survivors in Hiroshima, 1951–1985. Radiat. Res. **141**: 278–286.
13. Imaizumi, M., Usa T., Tominaga, T., Neriishi, K., Akahoshi, M., Nakashima, E., Ashizawa, K., Hida, A., Soda, M., Fujiwara,

S., Yamada, M., Ejima, E., Yokoyama, N., Okubo, M., Sugino, K., Suzuki, G., Maeda, R., Nagataki, S. and Eguchi, K. (2006) Radiation dose-response relationships for thyroid nodules and autoimmune thyroid diseases in Hiroshima and Nagasaki atomic bomb survivors 55–58 years after radiation exposure. J.A.M.A. **295**: 1011–1022.
14. Sakaguchi, S. (2003) The origin of FoxP3-expressing $CD4^+$ regulatory T cells: thymus or periphery. J. Clin. Invest. **112**: 1310–1312.
15. Ina, Y. and Sakai, K. (2005) Activation of immunological network by chronic low-dose-rate irradiation in wild-type mouse strains: analysis of immune cell populations and surface molecules. Int. J. Radiat. Biol. **81**: 721–729.
16. Hashimoto, S., Shirato, H., Hosokawa, M., Nishioka, T., Kuramitsu, Y., Matsushita, K., Kobayashi, M. and Miyasaka, K. (1999) The Suppression of metastases and the change in host immune response after low-dose total-body irradiation in tumor-bearing rats. Radiat. Res. **151**: 717–724.
17. Hayashi, T., Morishita, Y., Kubo, Y., Kusunoki, Y., Hayashi, I., Kasagi, F., Hakoda, M., Kyoizumi, S. and Nakachi, K. (2005) Long-term effects of radiation dose on inflammatory markers in atomic bomb survivors. Am. J. Med. **118**: 83–86.
18. Hayashi, T., Kusunoki, Y., Hakoda, M., Morishita, Y., Kubo, Y., Maki, M., Kasagi, F., Kodama, K., Macphee, D. G. and Kyoizumi, S. (2003) Radiation dose-dependent increases in inflammatory response markers in A-bomb survivors. Int. J. Radiat. Biol. **79**: 129–136.
19. Neriishi, K., Nakashima, E. and Delongchamp, R. R. (2001) Persistent subclinical inflammation among A-bomb survivors. Int. J. Radiat. Biol. **77**: 475–482.
20. Mariotti, S., Sansoni, P., Barbesino, G., Caturegli, P., Monti, D., Cossarizza, A., Giacomelli, T., Passeri, G., Fagiolo, U., Pinchera, A. and Franceschi, C. (1992) Thyroid and other organ-specific autoantibodies in healthy centenarians. Lancet. **339**: 1506–1508.

Received on June 11, 2009
Revision received on July 28, 2009
Accepted on July 31, 2009
J-STAGE Advance Publication Date: September 16, 2009