Short-time Induction of Thymocyte Apoptosis and Long-term Augmentation of Immune Responses by 50 cGy Gamma-irradiation to Mice

J. Matsubara¹, V. Turcanu², N. Sugiura ³ and T. Kosako³

¹Yokohama City University School of Medicine, Yokohama 236-0004, Japan
² Dept. of Pathology and Microbiology, Univ. of Bristol, Bristol BS8 1TD, UK
³Research Center for Nuclear Science and Technology, Univ. of Tokyo, 113-0032, Japan

INTRODUCTION

The scientists know that it is difficult to detect the statistically significant excess cancer death by epidemiological studies of people exposed at dose levels below 100 mSv. While the public want to know the effect of radiation even at very low doses. Therefore, LNT hypothesis was utilized since long to compensate the insufficiency of the knowledge. However, we believe that the scientific evidences obtained from the whole body experiments with animals on relatively low doses and their responses can supply considerable information apart from the chronic studies of cancer onset at a very low level which are difficult even with animal experiments. Nowadays we have a lot of evidences that cells respond to environmental factors (chemicals or radiation even at a very low dose) and are trying to analyze the mechanism at the molecular level. However, the data obtained from the whole body experiments are still scarce so that we discuss the dose-response issues from the biological point of view. With epidemiological studies we can estimate radiation risk but it is difficult to analyze or understand the effects of radiation due to confounding factors in human population. Without the evidences on the effect of radiation on the whole body(animal) public can not arrive at realistic understanding of the effect of radiation at low doses.

Our studies started to observe the dose-response curve of survival of the mouse under the varied environmental conditions since 1980(1). We observed the fact that there are many factors to modify the dose response curve and that these factors are directly related with the activation of host-defense function of animals(1)-(8). At a window of a certain dose-range animals could react and increase their radioresistance (9)-(11). It was proven by the present study that there was an augmentation of immune reaction after a specific time-lag after 5cGy or 50 cGy irradiation, it appears only at a very time when the mouse express the optimum increase of the survival against the damage from the lethal irradiation(5, 12).

MATERIALS AND METHODS

Mice and Pretreatments

Six week old male, specific pathogen free, C57BL/6 or ICR mice from Japan SLC(Hamamatsu) were used for experiments fed MF mouse chew from Oriental Yeast Co., Japan. Mice were irradiated in the Research Center for Nuclear Science and Technology, The University of Tokyo with a ¹³⁷Cs source having a dose rate of 5 cGy / min. Control mice were sham-irradiated in similar conditions. For the study of apoptosis 3 mice for each experimental group and 5-10 mice for each group of the PFC studies were assigned.

Apoptotic Cell Assay

The thymocyte apoptosis was determined using the Hoechst 33342 staining as described (12,13). Briefly, at different time points after irradiation, mice thymes were removed and a monocellular suspension in RPMI 1640 culture medium was prepared. Hoechst 33342 was added to the thymocyte suspension to a final concentration of 2.5 μg / ml, incubated at 37°C for 15 min. Cells having an apoptotic morphology of the nucleus (increased fluorescence, disappearance of chromatin structure) were identified by fluorescence microscopy. Three hundred cells from at least 10 microscope fields were counted and the apoptotic index was expressed as the percentage of apoptotic cells in the thymocyte suspension. Dexamethazone(DEX) was used as a positive control reaction (14). Splenocyte apoptotic index was determined in the same manner from the spleen of the experimental mouse.

Anti-SRBC PFC Assay

In order to count PFC, 5-10 mice from each group were immunized by injecting SRBC 15%(v/v) in saline at a dose of 0.02ml/ g body weight, i.p. at a fixed time interval after each pretreatment. Then 6 days after SRBC injection they were sacrificed and the spleens were removed. A monocellular suspension was prepared. Incubation mixtures were prepared with 10 μl spleen cell suspension, 50 μl of a 50% SRBC suspension, 50 μl of complement solution and 490 μl RPMI 1640. After gently mixing in a water bath at 37°C for 5 min, the suspension was plated in Cunningham chambers which were sealed with paraffin. After incubation for 1h at 37°C, triplicate PFC counts per mouse were performed(12).
RESULTS

With C57Bl/6 mice we found maximal levels of apoptosis 6h after irradiation, and thereafter the apoptotic index decreased and reached the background or sham-irradiated level 24h postirradiation. Therefore, we investigated the effect of irradiation on mice with doses of 7.5 cGy, 50 cGy and 300 cGy (=3Gy) by determining the levels of thymocyte apoptosis 6 h after irradiation. The result is shown in the Fig. 1. A significant increase of the apoptotic index in the thymus appears after 6h even with a very low irradiation dose of 7.5 cGy. Higher doses of radiation also induce higher levels of apoptosis and 3 Gy induce high levels of thymocyte apoptosis, similar to those induced by the injection of 1mg DEX / mouse. We measured thymocyte apoptosis with ICR mice in the same manner. It was confirmed that similar results were obtained from ICR mice(12).

![Fig. 1 Kinetics of the in vivo induction of apoptosis in C57BL/6 mouse thymocytes and splenocytes after irradiation. Each value expresses mean +/- standard deviation(SD) of triplicate counts from one mouse.](image1)

![Fig. 2 Plaque-forming cell (PFC) counts in ICR mice preirradiated with different doses. After different lag-times, mice were immunized by i.p. injection of SRBCs, 2 weeks after 50 cGy irradiation or 8 weeks after 5 or 10 cGy irradiation. PFC numbers were determined 4 days after immunization. Each bar shows mean PFC counts (x10^5 / spleen) +/- SD.](image2)

We studied immune reactions by counting spleen PFC with ICR mice immunized by SRBC. Results shown in Fig. 2 demonstrate prominent increases of PFC in mice subjected to one of the preirradiation treatments: 5 cGy or 10 cGy 8 weeks prior to SRBC injection, or 50 cGy 2 weeks prior to SRBC injection. In order to examine the kinetics of the activation of splenocytes time-course studies were performed using 30 mice of each strain (C57BL/6 or ICR) divided into 5 groups: 1) sham-irradiated and immunized with SRBC i.p. 2 weeks later; 2), 3), 4) and 5): irradiated with 50 cGy and immunized with SRBC at 1 day, 1 week, 2 weeks and 4 weeks respectively. Spleen PFC were counted 6 days after SRBC immunization. This time-course study established the effect of a single pretreatment (50 cGy irradiation) upon PFC counts with C57BL/6 and ICR mice respectively (See Fig.3). It is striking that the PFC counts measured two weeks after a 50 cGy
irradiation, are almost twice as high as those from sham-irradiated controls. However, if irradiated mice are immunized earlier (i.e. one day or one week after irradiation) or later (i.e. 4 weeks after the irradiation) there is no modification of PFC counts.

**DISCUSSION**

Mice were immunized with SRBC to assess both T cell and B cell activity as it was demonstrated that anti-SRBC PFC counts depend upon both functional T and B cells (15,16). It is generally accepted that anti-SRBC PFC counts reflects the T cell dependent anti-SRBC antibody production of B cells (16,17). This process is also accompanied by other effects upon the immune cells function, suggesting a specific increase in the body defense mechanisms, e.g. increase of hemolytic reaction titers in the sera of mice similarly pretreated (7).

![Fig. 3 PFC counts in sham-irradiated (control) or 50 cGy-preirradiated mice after different time courses of immunization with SRBCs.](image)

Yonezawa et al. (10,11) reviewed previous reports on the effect of whole body irradiation of low doses on the survival of mice and confirmed repeatedly that the promotion of radioresistance appears only at a specific time lag for each dose of irradiation, namely the optimum increase of survivals was observed about 8 weeks after 5-10cGy preirradiation and about 2 weeks after 50cGy preirradiation. Therefore, we investigated the biological effects of low doses of gamma irradiation by counting PFC after a specified period of time when the optimal radioresistance in mice was observed.

We compared also the effects of low dose radiation with other stress-inducing treatments which yield stronger radioresistance and liver MT synthesis at the same time (Mn²⁺, Cd²⁺, skin excision) (12). Different *in vivo* treatments induced a rapid increase in thymocyte apoptosis, peaking at 6h post-stress and decreasing to basal levels after 24h (10). We found that even such a low dose of radiation as 7.5 cGy induces a significant increase of thymocyte apoptosis and that the increase of radiation dose (50 cGy) also augments the percentage of apoptotic thymocytes in the thymus. A radiation dose of 3 Gy which has no hormetic effects induces almost complete thymocyte apoptosis, comparable to that induced by a high dose injection of DEX.

We also note that the effect of radiation upon thymocytes is short-lived. Indeed, although a relatively high percentage of thymocytes are committed to apoptosis due to the effect of the gamma rays, the overall level of thymic apoptosis returns to basal levels after 24h. Nevertheless, the global effects of low dose radiation are visible in a delayed manner, in a similar way as the radioprotective effects.

In our studies, the overall efficiency of the immune response, measured as PFC counts 4 - 7 days after
SRBC immunization, is increased only if SRBC were injected 2 weeks after 50 cGy of irradiation. On the contrary, if mice were immunized with SRBC one day or one week after the low dose irradiation or later than 3 weeks, no significant effect upon the intensity of the immune response could be seen. The lack of immediate or short-term effects of low dose irradiation upon PFC counts is also reflected by the lack of apoptosis induction by such 7,5 and 50 cGy irradiation in the spleens (12).

In the present study, we found that stress-inducing agents including LDR which generate delayed radioresistance act upon the immune system. Among the immune effects, we described the induction of thymocyte apoptosis and the increase of spleen PFC counts. Stress-MT inducing treatment seems to act within a few days stimulating more events at the biochemical level (1, 2, 8), while the low dose irradiation seems to work more at the cellular or at the whole body level influencing the cooperation between immune system components. We assume that the measured phenomena (i.e. radioresistance or increased immune response) are overall results from various coexisting processes which act at different levels of the body defense mechanism.

REFERENCES