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THYMOCYTE PROLIFERATION AND APOPTOSIS INDUCED BY IONIZING RADIATION

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Abstract

Proliferation and apoptosis of rat and mouse thymocytes caused by ionizing radiation were studied. The percentage of proliferating cells was determined by the method of colchicine metaphases and the apoptosis was estimated as DNA fragmentation. *In vitro* irradiation with 0.05-0.2 Gy was found to stimulate thymocyte proliferation, the maximum was observed at 0.05 Gy for mouse thymocytes and at 0.1 Gy for rat thymocytes. These doses caused a slight decrease in DNA fragmentation, as compared to control. By raising the radiation dose, proliferation was reduced and DNA fragmentation was increased. The results obtained indicate that low radiation doses stimulate cell proliferation while higher doses trigger apoptosis of thymocytes.

Key Words: Thymocytes, ionizing radiation, phorbol 12-myristate 13-acetate (PMA), proliferation, apoptosis.

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Introduction

Cell apoptosis and proliferation in the organism are controlled by different factors. Currently, there is evidence to suggest that, depending on dose, one agent may initiate both of these processes. Thus, it was found that growth factors added at concentrations above and below the optimal range, may result in cell apoptosis [2]. Although ionizing radiation was considered traditionally as a cell damaging factor, recent studies indicate that low doses of this agent stimulate proliferation of mammalian cells like cultured cells [6, 10], as well as peripheral blood lymphocytes [9], and splenocytes [7]. It is known that thymocytes are highly radiosensitive cells, and under moderate doses of radiation (1-4 Gy), undergo interphase death by the process of apoptosis [13]. The possibility of proliferation induction by low radiation doses was not studied in these cells. To verify this possibility, we studied the proliferation and apoptosis of thymocytes within a broad range irradiation doses, including the radiation doses reported to induce proliferation of other mammalian cells [4, 6, 7, 10].

Materials and Methods

Thymocytes were isolated from male Wistar rats (140-160 g) and male F_1 x C_{57} BL/6 mice (22-24 g) by standard procedure [5]. The cells were suspended in RPMI 1640 medium supplemented with 10% bovine serum, 10 mM HEPES. Part of each cell sample was irradiated with ^{60}Co gamma-rays at a dose rate of 0.14 Gy/min in doses ranging from 0.01-2 Gy at room temperature (the experimental protocol was identical for control and treated samples excepting irradiation). The postirradiation incubation was carried out at 37°C in 96-well flat-bottomed plates at a concentration of 1-2 x 10^7 cells/ml.

DNA fragmentation was determined by spectrophotometric assay of cleaved versus non-cleaved chromatin [11] in unirradiated (control) and irradiated thymocytes after a 6 hour incubation. We have previously shown [3] that 6 hours after irradiation the cells do not lyse and DNA content in the medium due to the lysis is insignifi-

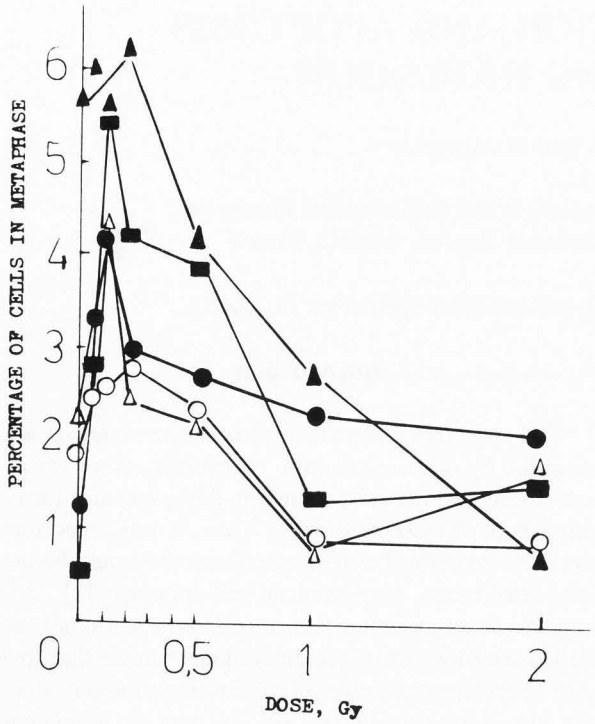

Figure 1. Radiation dose dependence of metaphase accumulation in rat thymocytes from various animals.

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cantly small, therefore, DNA fragmentation at this time can be reliably assayed. Another motivation is that fragmentation at this 6 hour time point corresponds to an intermediate value and can be modified either toward its minimum or maximum estimate [3, 5]. The cells (1.0 x 10^7) were harvested by centrifugation for 5 minutes at 700 x *g* lysed in 0.5% Triton X-100 containing 5 mM Tris-HCl (pH 8), 20 mM ethylenediaminetetraacetic acid (EDTA) for 15 minutes on ice. The samples were then centrifuged at 13000 *g* to separate high-molecular-weight chromatin (pellet) from cleavage products (supernatant). Pellets were resuspended in 1 ml of a buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Pellets and supernatants were assayed for DNA content using the diphenylamine reagent [1]. DNA staining with diphenylamine was performed at 30°C for 17-20 hours. DNA content was evaluated from absorption at a wave length of 600 nm. The percentage of fragmented DNA was determined as the ratio of the optical density in the supernatant to the sum of optical densities in the supernatant and the pellet.

Mitotic index of thymocytes was determined from the number of cells in colchicine metaphase after 6 hour incubation [8]. To do this, colchicine, which is known to accumulate cells in the cell cycle metaphase, was added at a concentration of $3 \cdot 10^{-5}$ M to the control, irradiated and PMA-treated thymocyte suspensions. Phor-

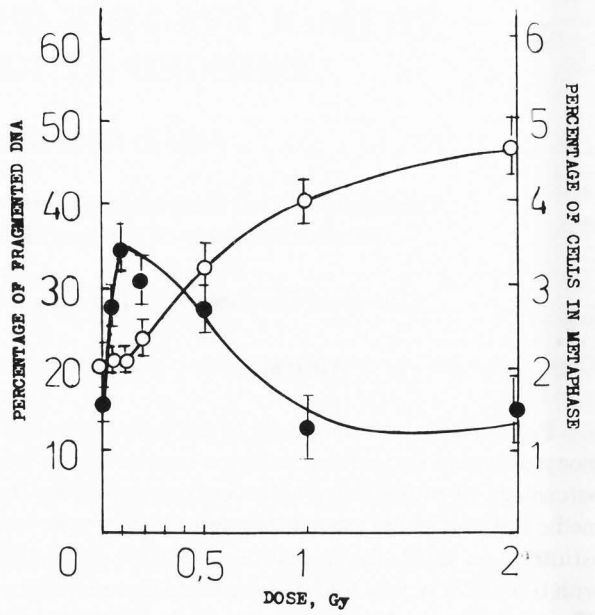

Figure 2. Radiation dose dependences of metaphase accumulation (•) and DNA fragmentation (○) in rat thymocytes, data are averaged between rats with low mitotic index in control thymocytes, n = 8. The difference between mitotic index in control and in variants irradiated with 0.1-0.5 Gy is highly significant ($p \leq 0.01$).

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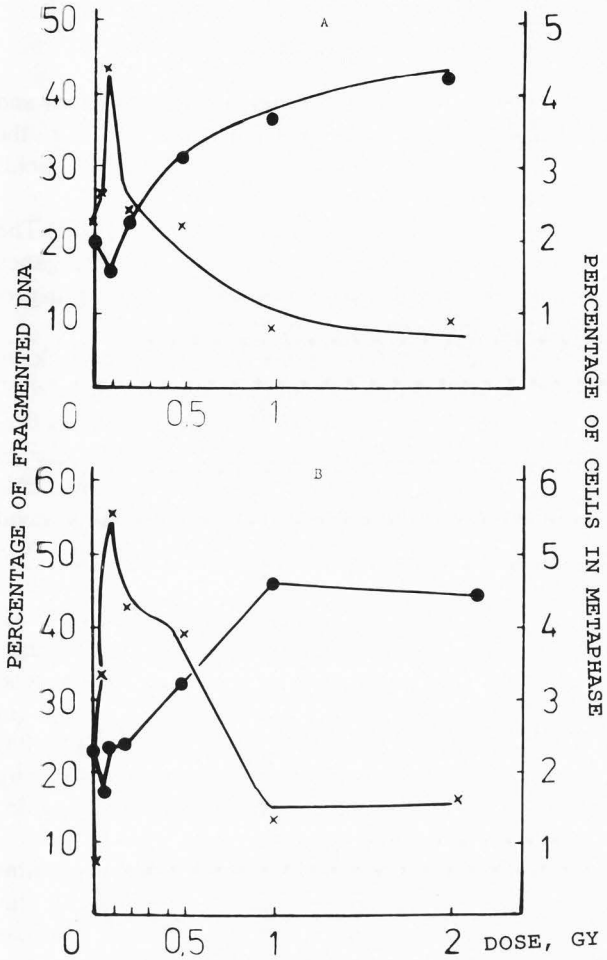
bol 12-myristate 13-acetate (PMA; Sigma, St. Louis, Mo, USA) was added immediately before incubation of cells at 37°C. Metaphase frequency was determined from visually counting of one or two thousand cells in each preparation. Points in the graphs are means ± standard deviation for triplicated or more ($n \geq 3$) experiments (n for each experiment is indicated in the figure's legend). The significance of the difference (P) between the control and low-doses treated variants was estimated by the Student's criterion.

Results

Figure 1 illustrates the dose dependence of accumulation of cells in metaphase for the rat thymocytes isolated from five individual animals. Low radiation doses, 0.05-0.1 Gy, stimulate thymocyte metaphase formation. The magnitude of the radiation effect is greater when control thymocytes in metaphase, which varies considerably among individual animals, is low. The maximum accumulation of cells in colchicine metaphase occurs after a dose of 0.1 Gy. Further increase in dose leads to a decrease in the frequency of cells in metaphase, and at doses of 1-2 Gy, this response falls below the control level. The decrease in the frequency of metaphase cells with increasing radiation dose is likely to be due to cell death (apoptosis). This is confirmed by comparing the



Figures 3A and 3B. Radiation dose dependences of metaphase accumulation (x) and DNA fragmentation (•) in rat thymocytes from individual animals.

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radiation dose dependence of thymocyte proliferation and the appearance of soluble DNA (Fig. 2). As shown in Figure 2, the decrease in cell proliferation is accompanied by an increase in DNA fragmentation. Based on the averaged dose dependence curve, DNA fragmentation remains at the control level in the range of low radiation doses (0.05-0.1 Gy). However, selected experimental data for individual animal thymocytes with low control mitotic index, showed a marked stimulatory effect in the mitotic index in the range of low radiation doses. The radiation dose dependencies of the cell death and proliferation responses are in counter-phase (Figs. 3A and 3B). Thus, the enhanced proliferation of rat thymocytes is accompanied by a decrease in DNA fragmentation, and vice versa. Similar results on stimulation of proliferation with low radiation doses were obtained for mouse thymocytes (Fig. 4). It is seen that the maximum of stimulation is shifted to the left, as compared to

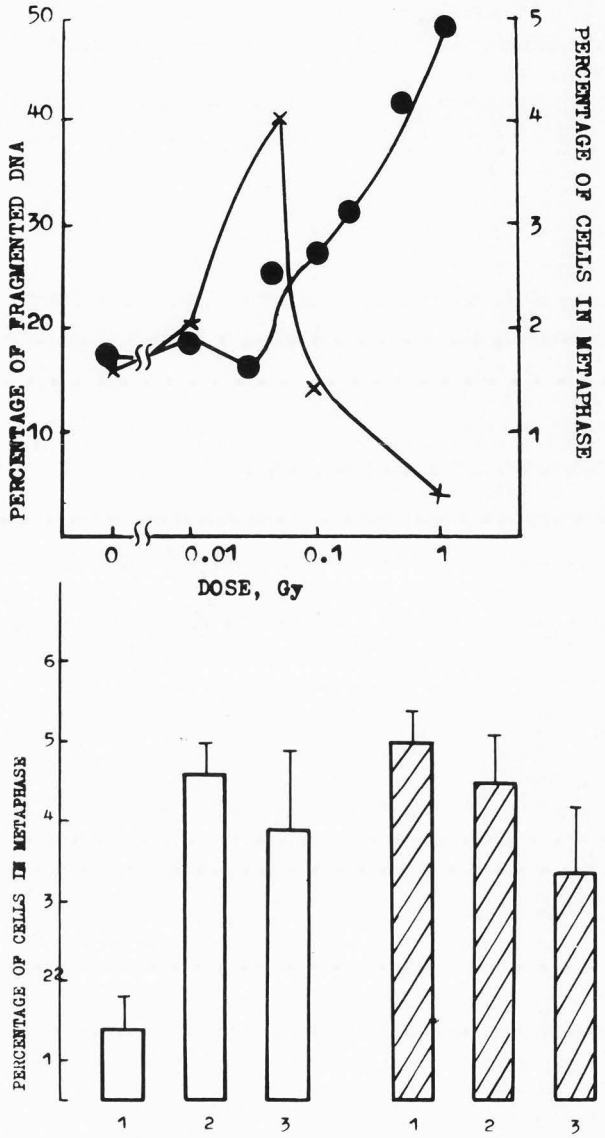

Figure 4 (top). Radiation dose dependences of metaphase accumulation (x) and DNA fragmentation (•) in mouse thymocytes (one typical experiment).

Figure 5 (bottom). Effect of PMA (10 nM) and irradiation (0.1 Gy) on metaphase accumulation of rat thymocytes isolated from animals with a low (open bars) and high (dashed bars) control level of thymocyte proliferation. n = 5. 1: control; 2: irradiation; 3: effect of 10 nM PMA. The difference between 1 and 2,3 (open bars) is highly significant with p value $\leq$ 0.01.

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rat thymocytes, and occurs at a dose of 0.05 Gy.

The relationship between the magnitude of the stimulatory effect of irradiation on the control mitotic index, shown in Figure 1, is likely due to the fact that, in the thymus, the proportion of cells capable of proliferating

is limited, and therefore, the higher the activated pool prior to radiation treatment, the lower the magnitude of the radiation effect must be. This assumption and the variability of the proliferating responses of individual animals to the agent simulating proliferation are confirmed by the experiments with the classic activator of cell proliferation, PMA. In Figure 5, the percent metaphase data averaged for two groups of animals is shown. Thymocytes from rats with a low control level of metaphase cells, the proliferation is stimulated by both exposure to 0.1 Gy and treatment with 10 nM PMA whereas thymocytes from rats with a high control level of metaphase cells, the proliferative response to irradiation is absent, and the percentage of metaphase cells in PMA-treated thymocytes is even below control (Fig.5).

Discussion

The results of this work indicate that low radiation doses stimulate proliferation of rat and mouse thymocytes. Thus, the proliferative response of thymocytes to low radiation levels is not different from that of other cells types investigated earlier. The optimal stimulatory radiation doses coincide for different cell types: 0.05-0.1 Gy for mouse and rat thymocytes (the present report), 0.1 Gy for Chinese hamster fibroblasts [6] and lymphoma cells [10], and 0.06-0.07 Gy for peripheral blood lymphocytes [4] and splenocytes [7]. The dependence of the magnitude of the stimulatory effect of radiation on the initial state of cell population shown in this work was also reported by Eliseeva *et al.* [4], who showed that, in animals with a high level of spontaneous blast transformation, low radiation doses stimulated insignificantly, if at all, the blast transformation of peripheral blood lymphocytes. Presumably, the reduction in the stimulatory effect of low radiation doses with increased control levels of metaphase cells, is due to the limited size of the proliferative pool in the thymus, and the variability of original proliferation level is related to the past history of the individual animal. It is known that the thymus responds to stress with the involution followed by regeneration [12]. These phases of thymus response to stress are characterized by significant changes in mitotic index, which reaches a maximum upon regeneration. Since the effect of stress varies even under laboratory conditions, the animals used in our experiment may have different past histories of stress and hence, different physiological state of the thymus. We also found that the dose dependences of proliferation and apoptosis are in counter-phase. These findings pose some problems of general and methodological character. One of them is the possible mutual distortion of the results: a decrease in DNA fragmentation due to cell proliferation and a decrease in proliferation due to cell death. Of great importance is a qualitative difference in the responses of cell populations (proliferation-death), depending on the intensity of the irradiating stimulus.

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