BIPHASIC PATTERN OF THYMUS REGENERATION AFTER WHOLE-BODY IRRADIATION*

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Heineke (1) and Rudberg (2) first described the effects of X-ray radiation on the thymus and noted the marked radiosensitivity of thymocytes. The effects of irradiation on the thymus in the rat, mouse, rabbit, guinea pig, and chicken have been classified into the following phases (3): (*a*) destruction, (*b*) aplasia, (*c*) proliferation of connective tissue, and (*d*) regeneration.

Using various strains of mice (ICR/Ha, CBA, C57Bl/6, and BDF_1), we have investigated the relationship of recovery of the thymus to radiation dose. We were surprised to find that whole-body irradiation with 300–400 Roentgens (R) caused a biphasic change in the weight of the thymus. Shortly after irradiation the weight of the thymus decreased and about 6 days later repopulation started, followed by a second apparently spontaneous, decrease in thymic weight. A secondary increase in organ weight started about 20 days after irradiation. If X-ray doses in excess of 500 R were employed, the first phase of recovery was minimal but the secondary increase in weight occurred at about the same time as before. If the X-ray dose was less than 200 R, the second phase of weight decrease did not occur.

Use of cytological techniques have made it possible to investigate the origin of cells repopulating the thymus. Experiments with thymus grafts (4–7), radiation chimeras (8–12), and parabionts (13, 14) showed that the thymus takes up lymphoid precursor cells from the circulation. In this study we have tried to elucidate the mechanism of biphasic regeneration of the thymus after whole-body irradiation.

Materials and Methods

Animals.—Male ICR/Ha (6-wk old), C57Bl/6, BDF_1 (C57Bl/6 × DBA/2)F_1, and CBA (10–12 wk old) mice were from the inbred colonies maintained in the Springville Laboratories.

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CBA/HT6T6 mice were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine. In these animals, skin grafts from CBA/Caj mice from the same laboratories took without rejection.

X-irradiation.—Mice were irradiated with a 250-kv General Electric Maxitron x-ray apparatus. The target-to-source distance was 50 cm, with 0.5 mm Cu and 1 mm Al filter, and the exposure rate was 60 R per min. The mice were housed in Perspex containers during the procedure. Hind legs of mice were shielded with lead (5 mm); the dose under the lead was measured as 2.5 R per min.

Histology.—Thymus tissue was removed immediately after sacrificing the animals, wet-weighed, and fixed in 10% formaldehyde. Standard histologic techniques were employed; hematoxylin-eosin was used for staining.

Determination of Mitotic Index.—The methods of Rothfels and Siminovitch (15) and Tjio and Puck (16) were used. Thymus tissue was fixed in 1:3 acetic methanol solution; 1 hr later, the tissue was stained with 2% acetoorcein solution, and squash preparations were made. About 20,000 cells were counted for each sample of the thymus, and the percentage of cells in the metaphase and early anaphase was calculated. Bone marrow cells were fixed with 50% acetic acid and stained with acetoorcein. Approximately 5,000 cells were counted for each sample.

Chromosome Analysis.—The methods used were those described by Ford, Hamerton, Barnes, and Loutit (8, 17). 2 hr after 0.3 ml of 0.04% Colcemid was injected into mice intraperitoneally, the animals were sacrificed and their femurs removed. By means of a syringe and needle, the bone marrow was flushed out with 1% sodium citrate solution warmed to 37°C, and was mixed well by repeated aspiration and discharge from the syringe. Thymus tissue removed immediately following sacrifice was teased in 1% sodium citrate solution. 15 min later, suspensions of marrow or thymus cells were centrifuged for 10 min at 1,500 rpm in a refrigerated centrifuge, and the pellets were fixed with chilled acetic methanol for an hour. Several drops of the fixed cell suspensions were placed on microscope slides previously dipped into distilled water and dried over a flame. The slides were stained with Giemsa's solution. Normal CBA cells or T6T6 cells in metaphase were scored.

Preparation and Injection of Bone Marrow Cells.—A marrow plug was flushed out of the femur by means of a syringe and needle into Hanks' tissue culture medium 199. After mixing by repeated aspiration and discharge the solution was filtered through a layer of gauze. The number of nucleated cells was counted under a microscope. The suspension was adjusted to contain 5×10^6 cells in 0.5 ml. This amount was injected intravenously into the tail vein of recipient mice 2–4 hr after irradiation.

RESULTS

Thymus weights of ICR/Ha mice subjected to 400 R whole-body irradiation showed a biphasic pattern of regeneration (Fig. 1). At each time period, 10 animals were sacrificed and the wet weights of the thymuses recorded. The figures indicate mean thymic weights and standard errors. Soon after irradiation, thymus weight rapidly decreased, but at the 6th–7th day, it started to increase. At the 12th day after irradiation, normal thymus weight was almost restored. A second decrease in thymus weight began at this time, and was followed by a second phase of recovery starting after the 23rd day. After irradiation with 200 R, the first phase of recovery was identical with that following 400 R, but no secondary decrease in thymus weight was observed. Relatively little regeneration was observed up to the 12th day after irradiation with 500

R, but the thymus weight started to increase 3 wk later (Fig. 1). Similar results were obtained after whole-body irradiation with 550 R.

Figs. 4–7 are histological pictures of the regenerating thymus (Fig. 4, normal; Fig. 5, 4th day after irradiation; Fig. 6, 12th day; Fig. 7, 22nd day; all after 400 R of whole-body X-ray exposure). At the 4th day (Fig. 5), the thymus has a very thin cortex, in which there are only sparsely distributed small lymphocytes. High magnification shows intensive mitosis at this stage. On the 12th day, the weight of the thymus has almost returned to normal, and histological examination (Fig. 6) reveals a large cortex with many dark-stained small lymphocytes. At the 22nd day, the thymus has undergone a second phase of weight decrease, and its histology (Fig. 7) shows that although the

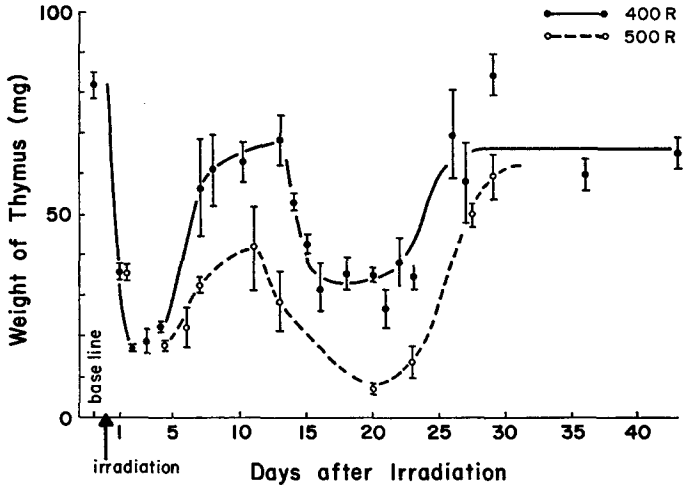

FIG. 1. Regeneration of thymic weight after whole-body X-irradiation of ICR/Ha mice

cortex is thin, it is larger and contains much more darkly stained small lymphocytes than during the first phase of involution (Fig. 5).

Fig. 2 shows changes in the mitotic index of the thymus after whole-body irradiation. Each point represents the index obtained from an individual mouse. After 400 R, the mitotic index fell to almost zero within 1 hr. It started to increase at day 3. At day 4, when the weight of the thymus was at the lowest, the mitotic index exceeded 1%. At the same time, much cell debris was observed. The index dropped rapidly to a level of little higher than normal and stayed at this level till day 14, at which time the second phase of weight drop has already been initiated.

These data seem to indicate that the first phase of thymus weight decrease was due to the direct damage to thymocytes by radiation (cessation of mitosis and death of cells), and the first phase of recovery was brought about by the

proliferation of the precursor cells remaining *in situ*. The regeneration curve following 500 R (Fig. 1) may suggest that most of the precursor cells died, thus permitting little regeneration up to the 12th day. This hypothesis explains the first increase in thymus weight, but does not explain the second decrease or the succeeding second phase of increase.

Fig. 3 shows the data of an experiment in which hind legs of BDF_1 mice were shielded during exposure to X-rays (400 R), or in which bone marrow cells (5×10^6) of a parental strain (C57Bl/6) were injected immediately after irradiation. Mean thymus weights of groups of 10 mice and standard errors are shown. The first decrease in thymus weight was not influenced by leg

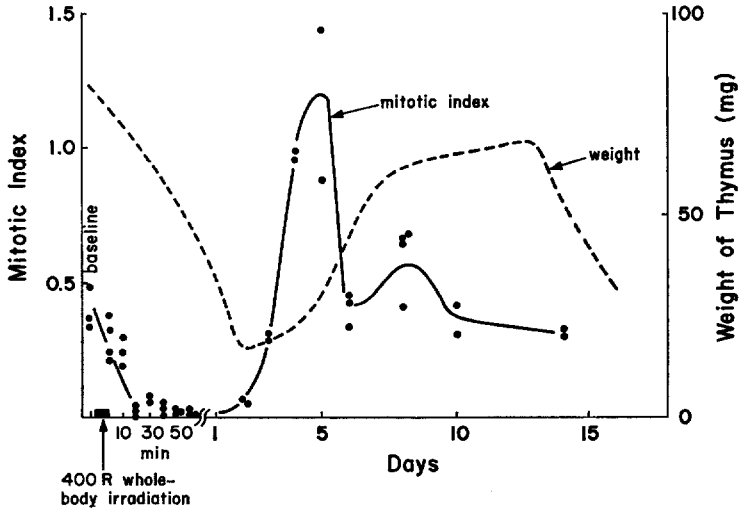

FIG. 2. Changes in mitotic index and weight of the thymus gland after whole-body irradiation (400 R) of ICR/Ha mice.

shielding, but no second decrease was observed. Injection of bone marrow cells shows similar effect to leg shielding, although it was less effective in preventing the second phase of thymus weight loss. These results indicate that the bone marrow injured by 400–500 R of X-rays does not contain as many thymus repopulating cells as are required for regeneration. There are several indications that the thymus may receive cells from outside sources (possibly the bone marrow). We hypothesize that the second decrease in thymus weight was due to impairment of the replacement of cells in the thymus.

In order to test this hypothesis, 5×10^6 bone marrow cells from CBA/HT6T6 mice were injected into CBA/Caj animals immediately after 400 R radiation. Bone marrow and thymus cells were harvested 10, 15, 22, and 29 days after irradiation. At day 10, more than 90% of the dividing cells in the bone marrow had T6T6 chromosome markers (Table I). Host cells, but not

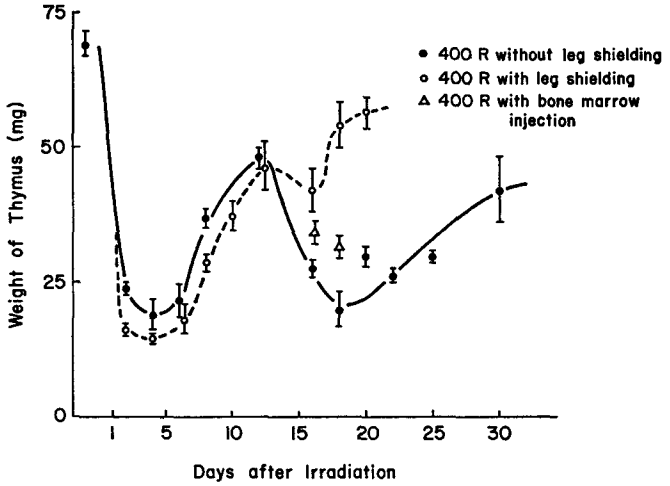

FIG. 3. Effect of whole-body irradiation (400 R) with hind legs shielded on thymic weight of BDF_1 mice. ○, the hind legs of the mice were shielded with lead during X-ray exposure (400 R). △, C57Bl/6 bone marrow cells (5×10^6) were injected immediately after irradiation (400 R).

TABLE I

CBA/Caj Mice Irradiated with 400 R and Injected with 5×10^6 CBA/HT6T6 Bone Marrow Cells

Time of sacrifice	Series	Mitotic cells scored					
		Bone marrow			Thymus		
		Host	Donor T6T6	T6T6	Host	Donor T6T6	T6T6
days				%			%
10	1	2	60	96.8	76	0	0
	2	6	63	91.3	65	0	0
15	1	32	63	66.3	13	90	87.3
	2	38	64	62.7	5	45	90.0
	3	16	75	82.4	33	71	68.2
22	1	48	50	51.0	6	70	92.1
	2	25	61	70.9	6	63	91.3
29	1	46	53	53.5	17	70	80.4
	2	13	51	78.4	10	64	86.4

CBA/HT6T6 donor cells, were found in the thymus. At day 15, some host cells appeared in the bone marrow but more than 80% of the metaphase cells in the thymus were of donor origin, indicating extensive replacement of the cells in the thymus by marrow-derived cells. On day 29, the majority of thymus

TABLE II

CBA/Caj Mice either Unirradiated or Irradiated and Injected with 5 × 10^6 CBA/HT6T6 Bone Marrow Cells — *Mitotic Index on Day 10 (without Bone Marrow Injection)*

Radiation dose	Series	Mitotic cells scored (bone marrow)			Radiation dose	Series	Mitotic index
		Host	Donor T6T6	T6T6			
R				%	*R*		
0*	1	72	0	0	0	1	0.52
	2	57	0	0		2	0.35
	3	57	0	0		3	0.26
						4	0.30
150‡	1	61	0	0			
	2	68	2	2.8	400	1	0.34
	3	58	3	4.9		2	0.29
	4	64	0	0		3	0.26
						4	0.27
400‡	1	2	60	96.8			
	2	6	63	91.3			

* Mice sacrificed 10 days after injection of T6T6 cells.

‡ Mice sacrificed 10 days after whole-body irradiation and injection of T6T6 cells.

cells and more than 50% of the dividing bone marrow cells had T6T6 markers. This seems to indicate that a continuous flow of marker cells from the bone marrow to the thymus occurred for a month after sublethal whole-body irradiation and marrow transplantation.

CBA/Caj mice were unirradiated or were exposed to 150 or 400 R, and 5 × 10^6 T6T6 bone marrow cells were injected intravenously 2–4 hr afterward. 10 days later, the mice were sacrificed, and the composition of the bone marrow cells was examined. Table II shows the results. All cells in the bone marrow of unirradiated CBA/Caj mice injected with T6T6 bone marrow cells were without chromosome markers, indicating that injected cells did not enter the bone marrow or proliferate there. CBA/Caj mice exposed to 150 R and injected with T6T6 cells had few marker cells in the bone marrow. The number of T6T6 cells in the marrow after exposure to 400 R was more than 90%. Thus, 150 R did not severely damage the bone marrow, and rapid recovery of host cells apparently prevented the entrance or proliferation of transplanted T6T6 cells in the bone marrow spaces. On the other hand, 400 R did damage the bone marrow, and permitted entrance and proliferation of T6T6 cells. The T6T6 cells that lodged in the bone marrow apparently suppressed the division of surviving host cells, resulting in few mitoses of the latter at day 10.

DISCUSSION

The high sensitivity of thymus cells to radiation was extensively studied by Murray (3), who found that the recovery of thymus tissue occurred around 1

wk after whole-body irradiation with 500 R and at 3 wk after 800 R. The present study showed that the thymus in mice of various strains exhibits a biphasic recovery after whole-body irradiation with 300–400 R. After 500 R or more, a slow, one-phase recovery pattern, involving mainly the second phase of regeneration, started 3 wk after irradiation. After 200 R, only the first phase of regeneration was observed.

The hypothesis that we wish to propose to explain our observations is as follows: (*a*) The first decrease in the weight of the thymus is caused by radiation-induced cessation of mitosis and death of cells. (*b*) The first phase of increase in thymus weight (peak at day 12) is due to proliferation of surviving precursors of small lymphoid cells in the thymus. (*c*) The second decrease in thymus weight (peak between days 18 and 22) is due to an insufficient supply of cells repopulating the thymus from the bone marrow, which was itself injured by irradiation. (*d*) The final regeneration of the thymus is due to recovery of the supply of cells repopulating the thymus from the bone marrow.

Regarding the first decrease, a decreased mitotic index following X-irradiation was found by Murray (3) as well as by us. The first phase of regeneration was shown to be due to an increase of cortical lymphoid cells. The mitotic index was high just before the first phase of increase in thymus weight. The fact that 500 R or more caused little first-phase recovery may be due to almost complete destruction of thymus cells that were able to divide after irradiation.

Dukor et al. studied the regeneration of thymus grafts (7), and showed that the pattern of regeneration of thymus tissue can be divided into four stages: (*a*) necrosis in the center of the grafted tissue during the 1st or 2nd day, (*b*) high mitotic activity, (*c*) reconstruction of the thymus by the 5th or 6th day, and (*d*) finally, by day 8, restoration of normal thymus architecture. Using chromosome markers, they showed that the cells dividing in the graft during the first 2 wk were derived entirely from donor cells surviving in the periphery of the original implant; by day 21, the dividing donor cell population had been entirely replaced by host cells.

This experiment and many others (4–6, 8–14) indicate that thymus cells are replaced by cells from outside the thymus gland. It takes about 3 wk for the complete replacement to be accomplished. Nothing is known concerning the identity of the cell types that migrate to the thymus under the conditions of the experiments.

Wu et al. (18) reported that marrow cells with radiation-induced chromosome markers, when transplanted into W/W^v anemic mice, were found in the thymus as well as the bone marrow of the host. When the marrow from mice with chromosome markers in the thymus was transplanted into irradiated recipients to form spleen colonies, the same marker present in the thymus was also found in many of the marrow-derived colonies (19). This suggests that at least some of the cells that repopulate the thymus may descend from the same stem cells that give rise to colony-forming cells.

Cudkowicz et al. found that the capacity of the bone marrow to repopulate the spleens of irradiated hosts is in proportion to its content of small and medium sized lymphocytes (20–23). These studies and those of Kielar and Mieneke (24) suggest that the cells responsible for recolonizing the marrow and spleen in irradiated animals are lymphocytes. Regarding the time of replacement of cells in the thymus after lethal whole-body irradiation, it has been shown that injected bone marrow cells (with chromosome markers) replace the cells in the thymus about 2–3 wk after X-irradiation (12, 25).

In our experiments, injection of bone marrow cells into irradiated mice partly prevented the second-phase decrease in thymus weight. Injected T6T6 bone marrow cells represented almost all of the dividing cells in the bone marrow at day 10 after irradiation but no T6T6 marker cells were found in the thymus at this time. At day 15, T6T6 cells started to appear in the thymus, and at day 22, most of the dividing cells in the organ contained chromosome markers. These results suggest that cells in the thymus are replaced by cells from other sources, and that the time of replacement coincides with that of the second decrease in thymus weight. It is quite natural that the thymus cannot obtain adequate numbers of cells if the organ that seeds it (most likely the bone marrow) is itself impaired by X-irradiation. The final recovery phase was observed 3 wk after whole-body X-irradiation. At this time, there may well be a sufficient supply of bone marrow cells destined to repopulate the thymus, because of the recovery of the bone marrow. The reason why there is no second-phase decrease in the experiments reported with thymus grafts (7) may be that the normal host bone marrow is functioning well, supplying sufficient numbers of repopulating cells to the thymus.

T6T6 marker bone marrow cells injected into control animals were not found in the bone marrow of the hosts, and only a few T6T6 cells were found in mice irradiated at a low level (150 R). On the other hand, almost all of the dividing cells in the marrow were of donor origin when the host animals were irradiated with 400 R. After nearly a month had passed, more than 50% of the dividing cells were still of donor origin. There may be a competition between transplanted T6T6 marrow cells and host cells in the bone marrow and later in the thymus. Ford et al. (26) implanted T6T6 marrow cells into mice whose legs were irradiated while the rest of the body was shielded. He found donor cells in the marrow cavities of the leg bones and later in the thymus, but not in unirradiated bones.

Recently, Micklem et al. (27) reported that T6T6 bone marrow cells (2×10^7) injected into normal CBA mice entered the bone marrow to a slight extent; the percentage of dividing cells in the marrow was 1.55% 1 wk after injection and 5.7% 12 wk later. The reason why they found some T6T6 cells in the bone marrow of CBA host mice, whereas we did not, may be that they injected a larger number of cells than we did (5×10^6). T6T6 cells were not

found in the thymus shortly after injection, but they started to appear later on.

The fact that injected bone marrow cells did not enter the thymus and proliferate immediately raises the following question: If cells seed from the bone marrow to the thymus continuously, why are *injected* bone marrow cells not able to enter and proliferate in this organ at the time of injection? The answer could be that the bone marrow cells that repopulate the thymus are different, in terms of in vitro viability, from those that recolonize lethally irradiated bone marrow soon after injection. They may be injured when they are taken from the donor, suspended, and injected into the recipient. Only the cells that colonize the bone marrow and spleen remain viable in bone marrow cell suspensions. Descendants of these cells may, however, seed the thymus, thus explaining the time delay in repopulating the thymus. An alternative explanation could be that the bone marrow does not contain cells that immediately repopulate the thymus. Nevertheless, some mechanism in the marrow permits certain cells to acquire the ability to repopulate the thymus. Cells may acquire this ability, for example, when crossing the marrow-blood barrier.

The fact that injected T6T6 marrow cells could enter heavily irradiated (400 R) bone marrow but not normal or lightly irradiated (150 R) bone marrow could be explained in this way: injected bone marrow cells compete for the opportunity to divide with those already residing in the marrow sinuses. When extremely large numbers of bone marrow cells are injected, or when host cell division is suppressed by X-irradiation, injected cells can lodge and divide in the bone marrow. Once grafted cells lodge in the marrow, they compete with host cells, interfere with their division, and persist in the marrow spaces even after the bone marrow cells of the host apparently recover the potential to proliferate. In fact, mitotic indices of the bone marrow 10 or 15 days following 400 R without injection of bone marrow cells were almost the same as those of normal mice (Table II), and thus, host bone marrow cells could have recovered their ability to divide and proliferate in the absence of grafted cells at those periods. Even so, more than 50% of the dividing cells in the bone marrow were of donor origin 10, 15, and 29 days after irradiation and bone marrow cell injection. A mechanism of feedback control could be responsible for the physiological regulation of the proliferation of hemic cells in the bone marrow.

SUMMARY

Whole-body irradiation of mice with 300 or 400 R causes a precipitous fall in thymus weight, followed by an increase in the mitotic index and an almost complete restoration of thymus mass. This phase is followed by a secondary fall in thymus weight and gradual recovery. This secondary fall can be prevented by intravenous injection of bone marrow or shielding of the hind limbs

during irradiation. The hypothesis is proposed that the thymus depends on the migration of cells from the bone marrow to the thymus for the maintenance of its cell population.

Bone marrow cells with chromosome markers injected intravenously into normal or lightly irradiated (150 R) animals do not populate the host bone marrow to any significant degree. After whole-body irradiation with heavy doses (400 R), donor cells dominate the marrow. There may be a competition between dividing cells in the bone marrow which regulates proliferation of hemic cells.

Bone marrow cells with marker chromosomes do not repopulate the thymus in irradiated animals until long after repopulating the bone marrow. It is possible that these cells have to pass through the marrow or the blood-marrow barrier to acquire characteristics needed for entering the thymus.

After whole-body irradiation with 500 R or more, the first phase of regeneration of the thymus, represented by an increase in the mitotic index, does not occur to a significant degree. Apparently cells in the thymus capable of proliferation have been largely eliminated, and restoration of organ mass depends chiefly on seeding from other sources, probably the bone marrow.

After whole-body irradiation with 200 R, only the first phase of thymus weight loss and regeneration takes place. Probably bone marrow injury is too small to interfere with the supply of cells repopulating the thymus.

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Addendum.—After this paper was submitted for publication, Dr. Peo Koller from the Chester Beatty Research Institute, London, England, visited us and mentioned that he and his collaborators have also observed a biphasic pattern of thymic regeneration after whole body irradiation. A. M. Cross, A. J. S. Davies, B. Doe, and E. Leuchars (1964, Time of action of the thymus, *Nature*, **203**:1239) speculated that the primary weight loss may be due to direct radiation injury and the secondary loss may be "related to the restoration of primary immunological responsiveness after irradiation". H. S. Kaplan, and M. B. Brown (1957, Radiation injury and regeneration of lymphoid tissues, *in* The Leukemias: Etiology, Pathophysiology and Treatment, J. W. Rebuck, F. H. Bethell, and R. W. Monte, editors, Academic Press, Inc., N.Y.) and G. Brecher, K. M. Endicott, H. Gump, and H. P. Browner (1948, Effects of X-ray on lymphoid and hemopoietic tissues of albino mice, *Blood*, **3**:1259) have also observed this phenomenon without offering an explanation for its mechanism.

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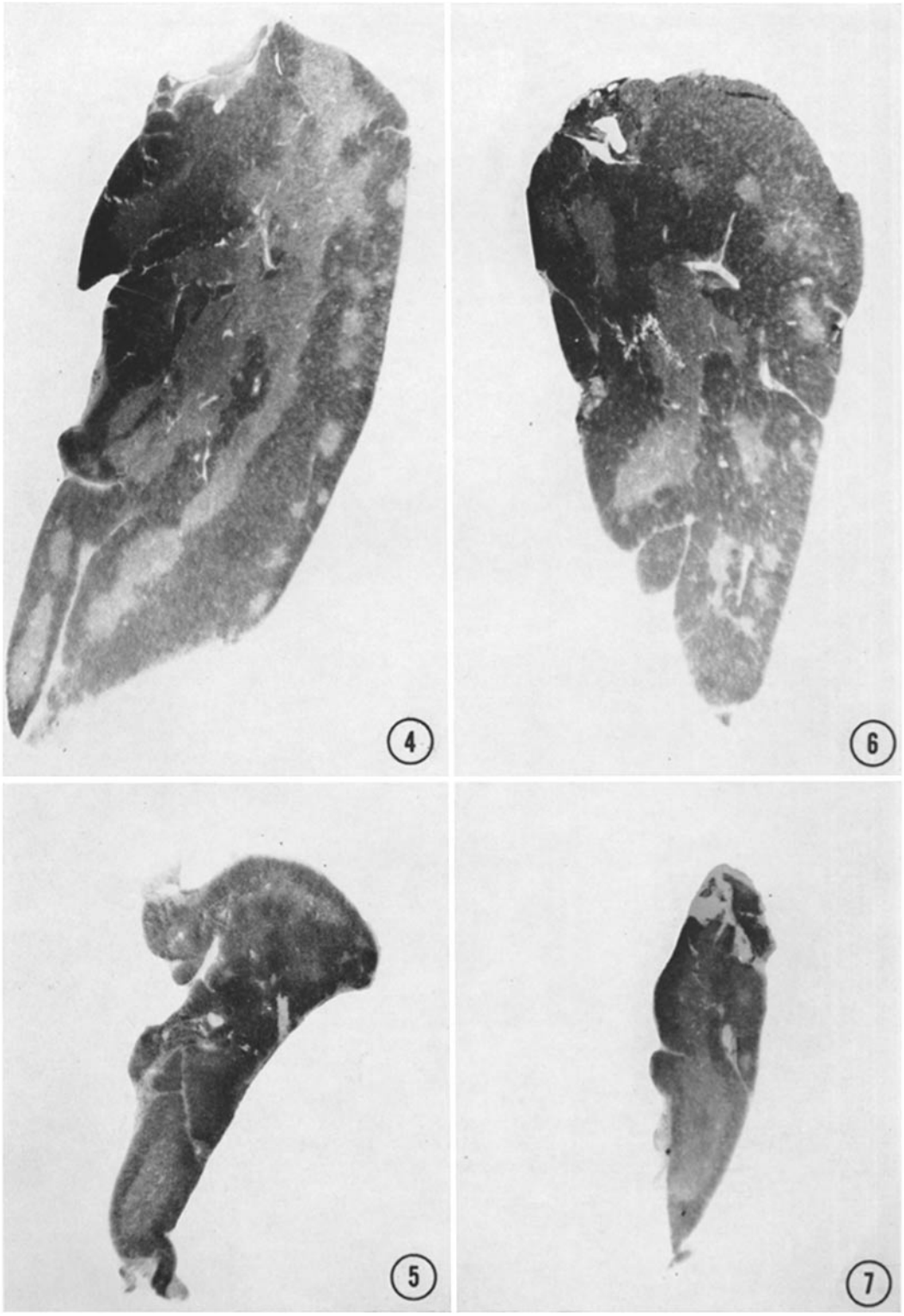
FIG. 4. Histological picture of normal thymus ICR/Ha mouse. Hematoxylin-eosin. × 15.

FIG. 5. Histological picture of thymus 4 days after whole-body irradiation (400 R) Hematoxylin-eosin. × 15.

FIG. 6. Histological picture of thymus 12 days after whole-body irradiation (400 R). Hematoxylin-eosin. × 15.

FIG. 7. Histological picture of thymus 22 days after whole-body irradiation (400 R). Hematoxylin-eosin. × 15.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