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Vascular and stromal changes in irradiated and recovering rat thymus

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Summary. To analyze the mechanisms responsible for thymocyte proliferation, maturation and migration in the thymus, the rat thymus just after, and recovering from irradiation was studied morphologically. The vascular structures of the rat thymus after a radiation dose of 6 Gy were found to be destroyed on day 3, but had recovered to almost normal by day 7, suggesting that the abrupt recovery of thymus structure after irradiation was due primarily to this change in vascular structure. Furthermore, the epithelial tissues in the thymic cortex appeared to contribute to this abrupt proliferation, and possibly to the abrupt maturation of thymocytes, while medullary epithelial tissues remained sparse and appeared inactive for a relatively long period. These findings are considered important for understanding the interrelationship between thymic epithelial cells and thymocytes with respect to thymocyte proliferation, maturation and migration.

Key words: Thymus, Irradiation, Proliferation, Vascular structure, Reticular epithelial cells

Introduction

After irradiation the thymus loses its unique structure. The number of thymocytes decreases rapidly and the cortico-medullary boundary disappears. However, these changes are rapidly repaired following abrupt proliferation of thymocytes (Tsuchida et al., 1994; Fujikura et al., 1997). We have been interested in this abrupt regeneration of thymocytes, and have been trying to clarify the mechanisms responsible. Initially, we analyzed the differences in phenotypic expression of thymocytes prepared from normal and irradiated but recovering thymuses. The percentages of immature-type thymocytes increased, and gradually recovered to the normal level (Konishi et al., 1994; Tsuchida et al., 1994).

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We have also reported the BrdU uptake of thymocytes after irradiation. Increase in the frequency of BrdU-positive cells was observed on days 5, 7 and 10, providing supportive evidence for the intensive proliferation of thymocytes after irradiation (Fujikura et al., 1997). Recently, we have also analysed the cellular interactions between allogenic lymphocytes and thymus cells in rat thymus following sublethal irradiation (Ohba et al., 1997).

In the present work, our aim was to study the mechanisms responsible for the abrupt proliferation of thymocytes. First, we analyzed the changes in the vascular structures, since the blood supply to thymic tissues might be important in this respect. To visualize the vascular structures in the thymus, India ink injection methods were employed. As well as the vascular changes, immunohistochemical analyses using monoclonal antibodies against rat MHC antigens and other monoclonal antibodies against rat cells were employed to analyse the changes in stromal tissues, including epithelial reticular cells, macrophages and dendritic cells, in the thymus.

Materials and methods

Experimental conditions

Female DA strain rats weighing 200 g were used. The rats were subjected to total body irradiation using an X-ray machine (MBR-1520, Hitachi Medical Co. Ltd., Tokyo, Japan). Each rat was placed in a thin-walled box, measuring 10x10x7.5 cm, and placed in the center of the room. The total irradiation dose was 6 Gy, delivered at a dose rate of about 1.9-2.0 Gy/min. The animals were irradiated through filters made of 0.5 cm-thick aluminum and 0.1 cm-thick copper. After irradiation, the rats were sacrificed on days 1, 3, 5, 7, 10 and 14 .

India ink injection

Rats were anesthetized with diethyl ether. They were then perfused with saline, followed by India ink mixed with gelatin. The thymus obtained was cooled on ice for

2 h, and sectioned with a slicer at a thickness of 200 μm. The vascular structures were observed by light microscopy.

Immunohistochemical staining

Animals were sacrificed by exsanguination under diethyl ether anesthesia. The thymus tissues were removed immediately, embedded in O.C.T. compound (Miles, Elkhart, IN, USA) and frozen, and 6 μm sections were cut on a cryostat at -20 °C. The sections were air-dried on microscope slides for 30-60 min at room temperature and fixed by immersion in ice-cold acetone/methanol (1:1) for 10 min. After washing three times in ice-cold phosphate-buffered saline, thymus sections were reacted with mouse monoclonal antibodies against rat antigens: OX6 (anti- MHC class II) , ED1 (anti-macrophage), ED2 (anti-macrophage), ED3 (anti-macrophage) and UB13 (anti-brain-thymus antigen) (Kuniki et al., 1995). Sections were then reacted with HRP-conjugated goat F(ab')2 anti mouse IgG antibody for 1 h at 4 °C. The antigens were visualised using 3-3' diaminobenzidine tetrahydrochloride, and the tissue sections were counterstained with hematoxylin. Polyclonal rabbit anti-FTS serum (kindly donated by Dr. T. Kakegawa, Department of Biochemistry, Faculty of Pharmaceutical Sciences, Chiba University, Japan) was also used. In this case HRP-conjugated goat $F(ab')_2$ anti-rabbit IgG antibody was used as a second antibody.

This experiment was reviewed by The Ethics Committee for Animal Experimentation of Yamaguchi University School of Medicine, and carried out according to the Guidelines for Animal Experimentation of Yamaguchi University School of Medicine and The Law (No.105) and Notification (No.6) of the Japanese Government.

Results

Vascular structures in the thymus

The thymus has a unique vasculature under normal

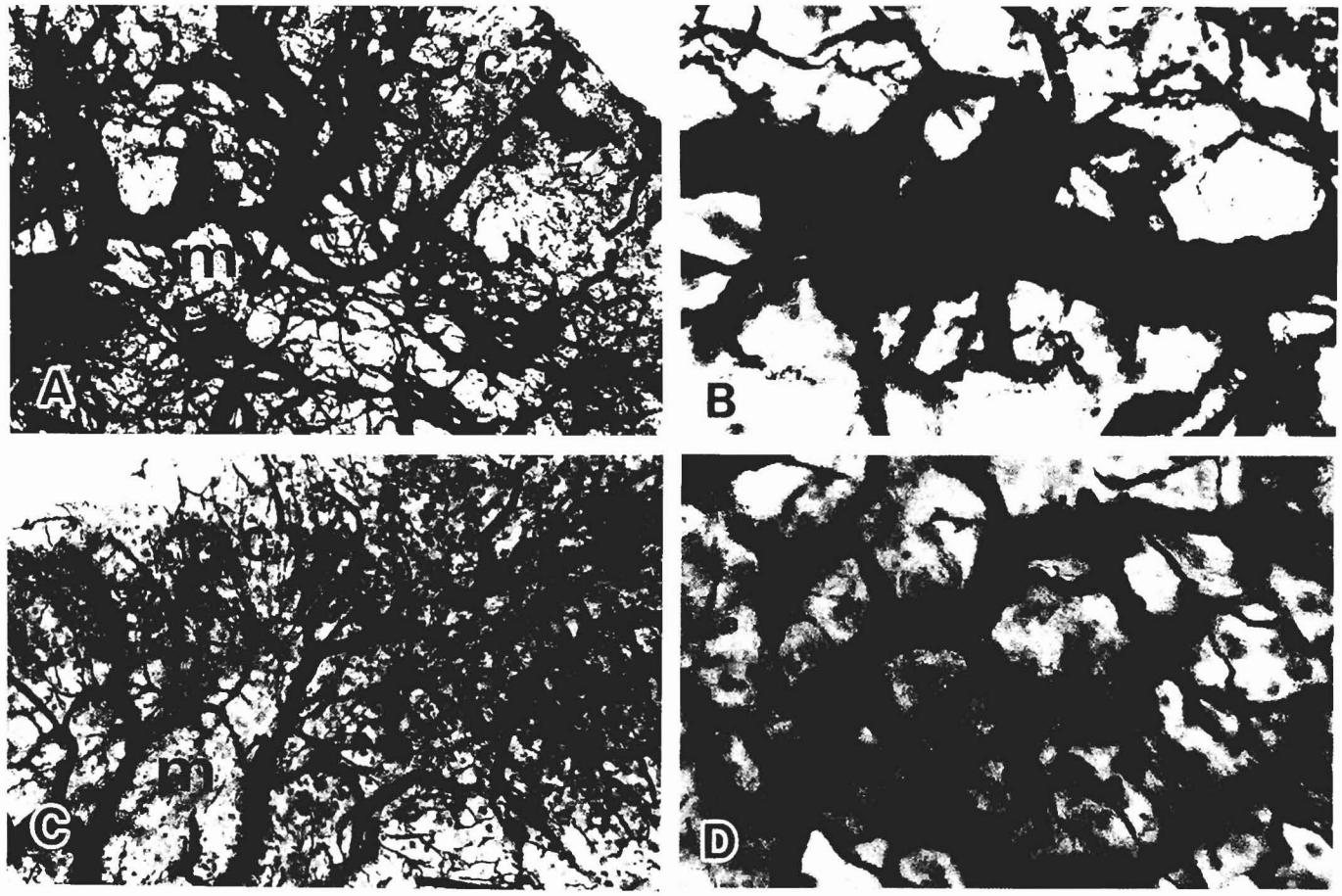

Fig. 1. Vascular structures in rat thymus after irradiation visualized using India ink injection. The vascular structures appear to have been destroyed on day 3 after irradiation **(A)**, and the vessels in the medulla are larger than normal with non-smooth edges **(B)**. On day 5, the fine vascular meshwork in the cortex appears to have been reestablished **(C)** and the edges of the vessels in both the cortex and medulla appear quite smooth **(D)**. m: medulla; c: cortex. A, C, x 72; B, x 180; D, x 360

conditions. A very fine capillary meshwork can be observed only in the cortical area, while in the medullary region vascular structures appears straight and rather larger than cortical vessels. The medullary vessels are extensions of thymic vessels supplying blood through the interlobular connective tissue. Through the capsular region, blood vessels are also supplied from the thymic artery or vein.

The fine vascular structures which were usually found in the cortex of the thymus appeared to be destroyed on day 3 after irradiation , while the vasculature in the medulla appeared larger in size, and the edges of the vessels in the medulla appeared to have lost their smooth outline (Fig. 1A, B). However, on day 5, the fine vascular meshwork in the cortex appeared to have been reestablished (Fig. 1C). At higher magnification, the vascular edges in both the cortex and medulla appeared quite smooth (Fig. 1D), suggesting recovery from damage to the vascular wall evoked by the radiation.

Immunohistochemical staining

Immunohistochemical staining was conducted on normal and irradiated but recovering thymuses using anti-rat monoclonal antibodies: OX6 (MHC class II molecule), ED2 (macrophage specific), and ED3 (macrophage specific). Polyclonal rabbit anti-FTS serum was used.

Fig. 2 shows the typical staining pattern of OX6 on normal rat thymus where medullary regions were heavily stained, while the thymus 3 days after irradiation showed strong staining in the cortex and rather weak staining in the medulla (Fig. 2B). ED2 and ED3 stained the cortical regions of the irradiated and recovering thymus extremely strongly and frequently, particularly on day 3 (Fig. 3B, E). These typical staining patterns changed to almost normal by day 7 (Fig. 3C, F). ED2 stained cortical macrophages in the normal thymus, while ED3 stained macrophages mainly residing in the corticomedullary region of the normal thymus (Fig. 3A, D). Anti-FTS polyclonal antibody strongly stained cells which were not thymocytes, but possibly reticular epithelial cells and fibroblasts, in the day 3 thymus (Fig. 4C, D). On the other hand, the thymus from the normal rat was weakly stained with this antibody (Fig. 4A).

Discussion

The effects of sublethal whole-body irradiation (6 Gy) on the rat thymus was examined using India ink analysis to reveal vascular changes, and by immunohistochemical staining using antibodies against rat tissues and FTS. The study was extended to thymus tissues recovering from irradiation, as we were interested in the mechanisms responsible for the abrupt proliferation of thymocytes, having previously reported the phenotypic changes in thymocytes and BrdU uptake by these cells (Konishi et al., 1994; Tsuchida et al., 1994; Fujikura et al., 1997; Fukumoto, 1997). The present results suggest that vascular repair, especially in the cortex, might play an important role in the rapid

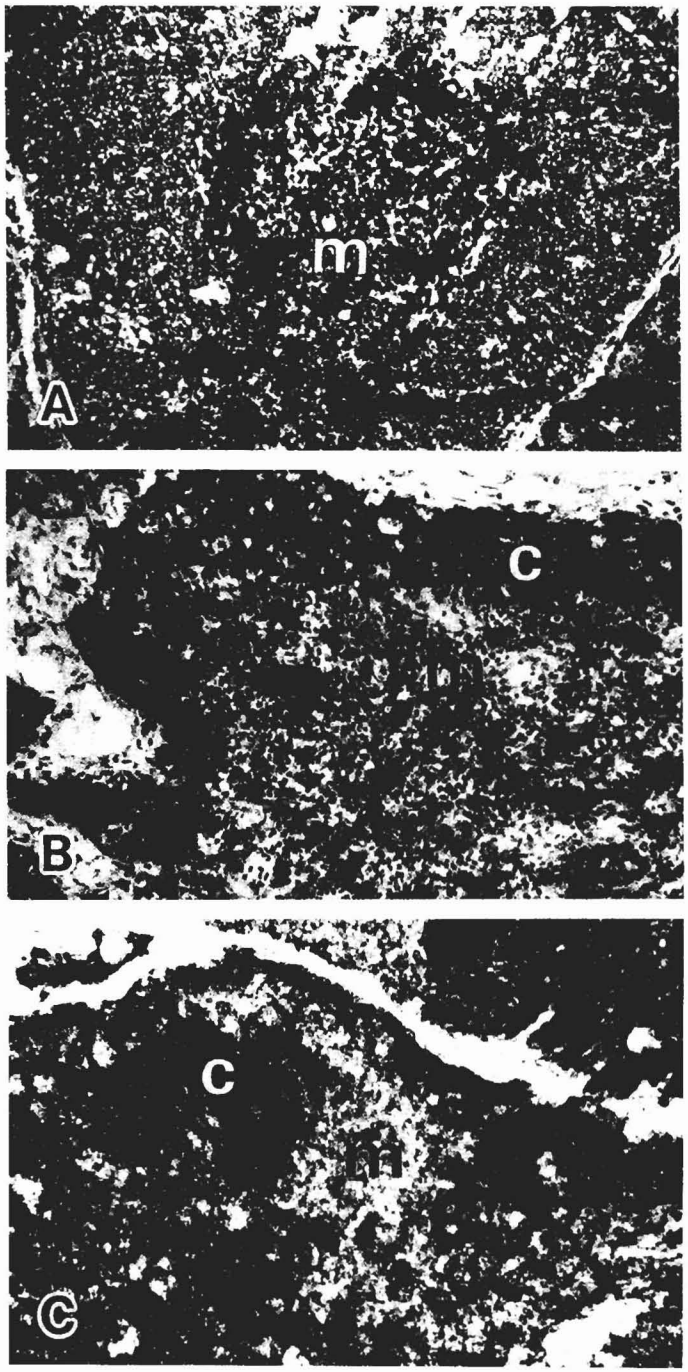

Fig. 2. Sections of thymus stained immunohistochemically with OX6 (MHC class II) on days 14 **(A)**, 3 **(B)**, and 7 **(C)** after irradiation. On day 14, the thymus has almost become reconstituted to a normal situation, with staining mainly in the medullary region (A). However on day 3 there is strong staining in the cortex and rather weak staining in the medulla (B). m: medulla; c: cortex. x 180

recovery of the thymus after irradiation (Fig. 1). Unique immunohistochemical staining was observed with anti-MHC class II antibody (OX6) (Fig. 2) and anti-macrophage antibodies (ED2 and ED3) (Fig. 3), being rather strong in the cortical region compared with the medulla. This suggests that some important process of cell dynamics may take place in the cortex during recovery of the thymus after whole-body irradiation.

In fact in our previous studies we found cells that were strongly TUNEL positive during recovery from

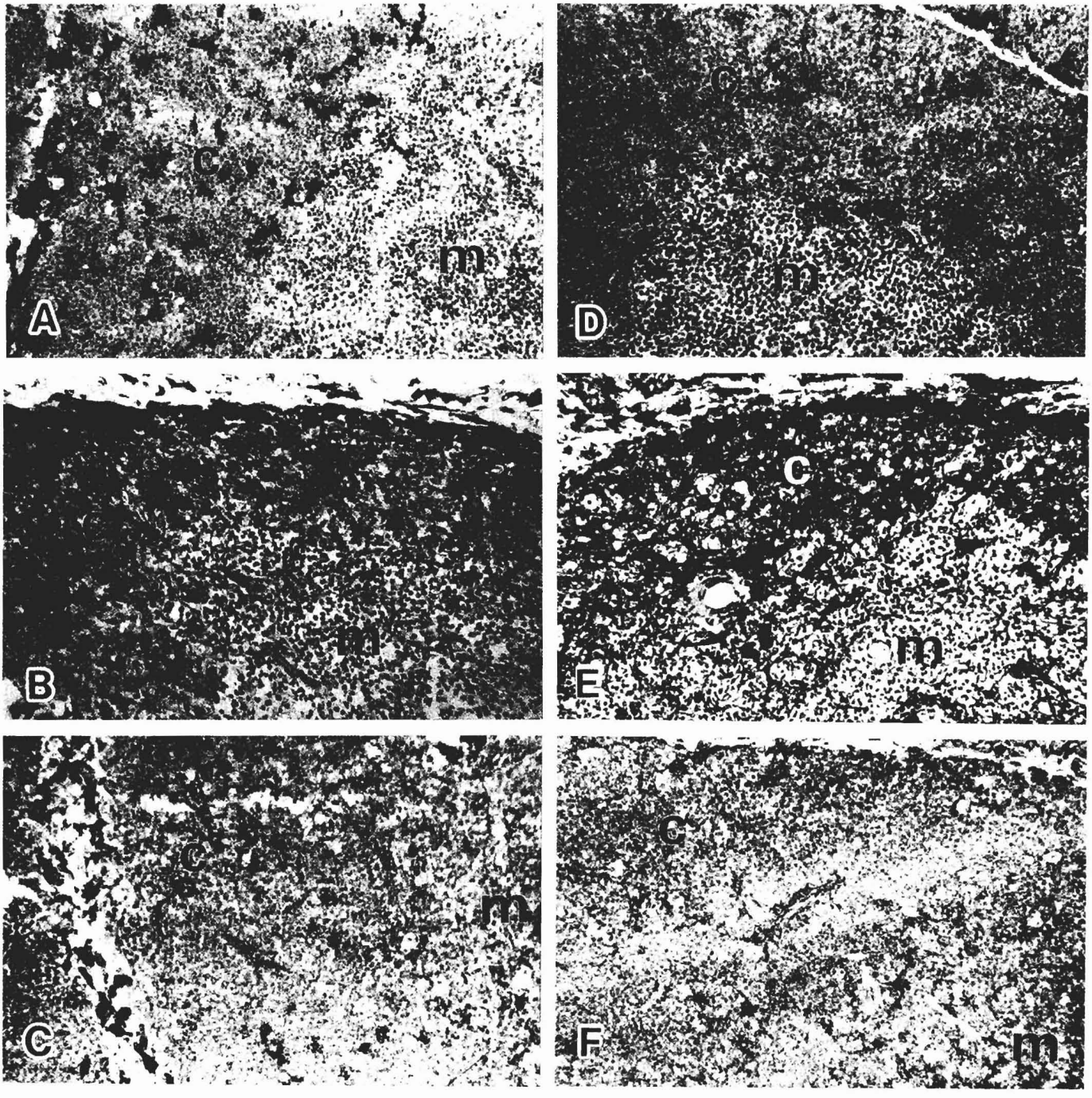

Fig. 3. Sections of thymus stained immunohistochemically with ED2 (macrophage- specific) and ED3 (macrophage-specific) on days 14, 3, and 7 after irradiation. ED2 stains cortical macrophages on day 14, as in the normal thymus **(A)**, while ED3 stains macrophages mainly in the corticomedullary region **(D)**. Both ED2 and ED3 show extremely strong staining in the cortical regions of the irradiated and recovering thymus, particularly on day 3 **(B, E)**. These typical staining patterns changed to almost normal by day 7 **(C, F)**. m: medulla; c: cortex. x 180

radiation damage in the cortex. This suggests that cell changes occurring in the cortex may be removed by the many macrophages present in this region and thus thymocytes were repaired abruptly. Cytokines secreted by macrophages such as IL-1 or TNF may be involved in cell proliferation and regulation existing in the thymus. Cells taking up BrdU are also numerous in the subcapsular region of the regenerating thymus (Fujikura et al., 1997). This extensive proliferation may be supported by MHC class II-positive epithelial reticular cells and FTS-positive cells. Further studies using cytokine analyses either at the molecular level or gene level using RT-PCR or in situ hybridization are required. We are now studying the changes in cytokine expression in irradiated and recovering thymuses. With respect to the unique staining observed for MHC class II and macrophages, further analyses are now being planned using new monoclonal antibodies against rat epithelial tissues. When we immunized mice with thymus tissue obtained 5 days after whole-body irradiation and tried to establish hybridomas, several potentially interesting monoclonal antibody-producing clones were established. One of these antibodies labeled the vascular structures of day 5 thymus, and stained the normal thymus weakly. Another antibody strongly labelled the cortex of the day 5 thymus, but did not stain the medulla, while in normal thymus, this antibody labelled the medulla strongly but the cortex weakly. Characteristics of this latter antibody were similar to the staining patterns obtained with OX6 (Wang, in preparation). These data support the findings of Adkins et al. (1998) that medullary epithelial tissues recovered less well than cortical epithelial tissues after irradiation. With respect to the differences between cortical and medullary epithelial cells, Pavlovic et al. (1993) have reported two interesting monoclonal antibodies that recognize epitopes on rat thymic cortical and medullary epithelial cells. We are now further studying the differences in morphological changes between cortical and medullary epithelial tissues in irradiated and recovering thymuses using immunoelectron microscopy.

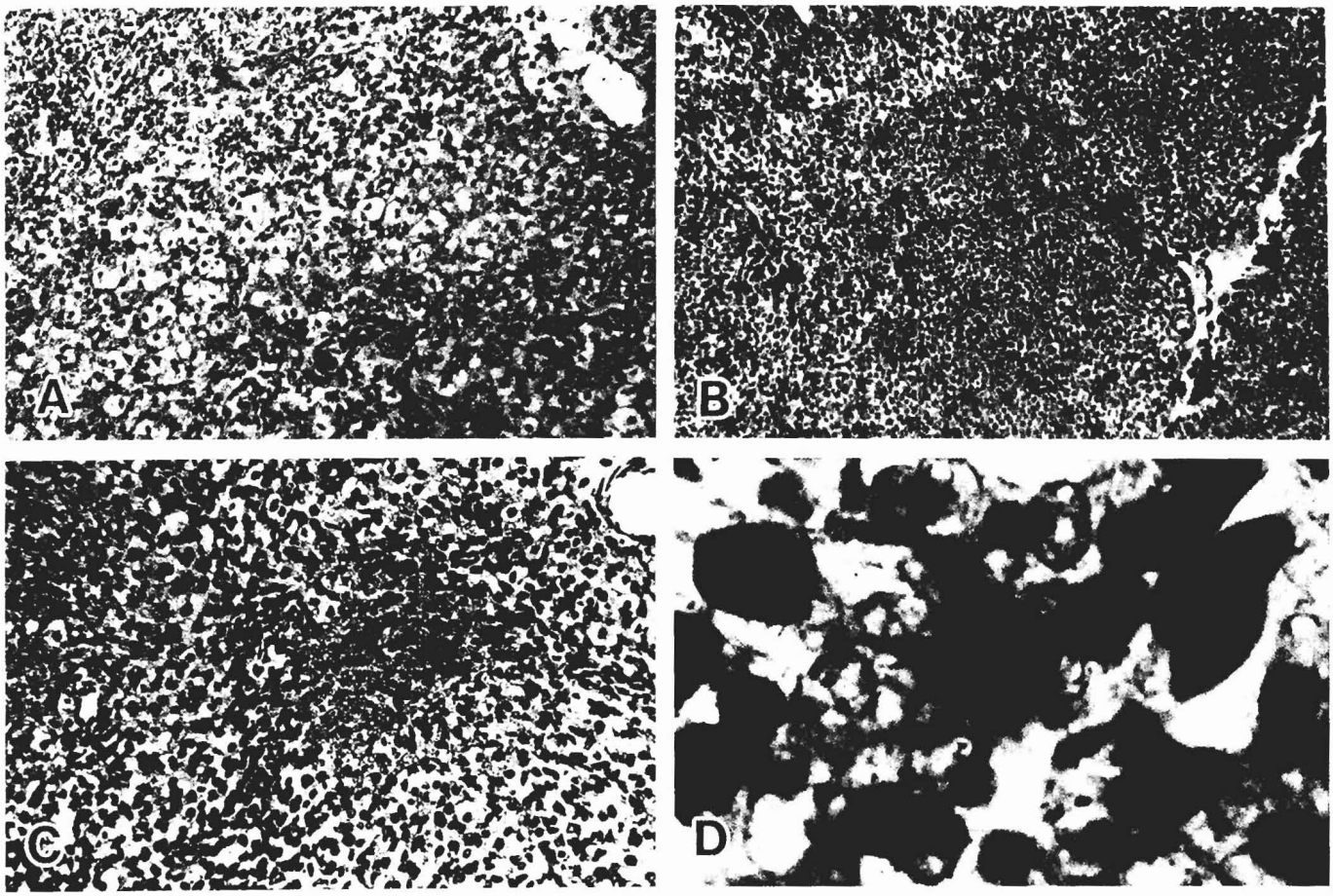

Fig. 4. Immunohistochemical staining with anti-FTS polycolonal antibody in the normal and irradiated but recovering rat thymus. Normal thymus is weakly stained with this antibody **(A)**. On day 3 **(C)** and 7 **(B)**, anti-FTS antibody stains the cells strongly. These are not thymocytes, but may be reticular epithelial cells and fibrocytes **(D)**. A, B, C, x 180; D, x1,800

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