Histol Histopath (1994) 9: 281-286

Histology and Histopathology

Morphometrical changes in the rat thymic lymphoid cells after treatment with two different doses of estradiol benzoate

A. Martín, L. Alonso, M. Gómez del Moral and A.G. Zapata
Department of Cell Biology, Faculty of Biology, Complutense University, Madrid, Spain

Summary. In the present study we have evaluated morphometrically the contribution of thymocytes to the thymic involution induced by a single injection either of 100 μg or 500 μg of estradiol benzoate. Our results demonstrate that changes in the numbers of both cortical and medullary thymocytes contribute to thymic involution although the importance of the first is quantitatively higher. On the other hand, while cortical pyknosis and a decreased mitotic index could be important for explaining the estrogen-dependent thymic changes, the release of lymphocytes from thymus seems to be the main factor inducing the thymic involution as well as the lack of recovery observed at the end of the experimental period.

Key words: Thymus, Sex steroids, Thymic epithelial cells, Macrophages, Thymic vascular permeability

Introduction

The thymus has been claimed to play a role in governing the reported effects of estrogens on the immune system (Grossman et al., 1982), although available data are scarce and a quantitative analysis is, as far as we know, lacking. Most authors, however, have observed thymic involution after estrogen injection, although its origin is a matter of discussion. Apart from some recent data from our group (Fonfría et al., 1983; Zapata et al., 1983; Leceta et al., 1989; Martín-Moreno, 1992) and others (Clark and Kendall, 1989) emphasizing the importance of thymic non-lymphoid cell components in the estrogen-dependent thymic involution, most authors relate it to either decreased number of cortical thymocytes (Shobon and Jirasatthan, 1974; Screpanti et al,, 1982; Kuhl et al., 1983; Luster et al., 1984) or both cortical and medullary lymphoid depletion (Leceta et al., 1988). Moreover, while some authors correlated thymic atrophy to a gradual loss of cortical lymphocytes without increasing number of pyknotic cells (Simmons, 1964; Luster et al., 1984), others mention abundant pyknosis in the involuted thymuses (Kalland et al., 1978) together with a decreased mitotic index (Mysliwska, 1979). Recently, Aboussaquira et al. (1991) correlated this inhibition of lymphocyte proliferation with an impaired T-cell maturation. In order to confirm how changes in thymic lymphoid cells affect estrogen-dependent thymic involution we have morphometrically studied the variations which occur in both cortical and medullary thymocytes of adult rat thymus after a single intraperitoneal injection with two doses of estradiol benzoate (EB).

Offprint requests to: Prof. A.G. Zapata, Department of Cell Biology, Faculty of Biology, Complutense University, 28040 Madrid, Spain

Materials and methods

Experimental procedure

Three-month-old female Wistar rats were subcutaneously injected with either 100 or 500 μg of EB dissolved in 0.1 ml of corn oil. Controls received the oil injection only. At 2, 7, 15 and 21 days after treatment, rats were sacrificed by decapitation, body weights determined, and the thymus removed, weighed, and processed for routine light microscopy. Briefly, the right lobe of the thymus of each rat studied was fixed in Bouin solution, dehydrated in alcohol and embedded in paraffin. Each thymic lobe was wholly sectioned and the obtained 8 μm-thick sections were stained with Alcian blue-Masson's haemalum-picroindige carmine. In addition, blood samples were collected and plasma used to determine estradiol concentration by radioimmunoassay (data not shown, see Martín-Moreno, 1992). Three rats per group and day were analyzed in the study.

Morphometrical analysis

On 10 different randomly-selected sections (by lobe and rat) routinely processed for light microscopy, the following parameters were determined: (a) relative areas

occupied either by cortex or medulla; (b) thymic cortex and medulla weight; (c) frequency of cortical and medullary lymphocytes; (d) percentage of cortical and medullary pyknotic cells; and (e) cortical and medullary mitotic index.

Cortical and medullary areas were determined using a planix-7 planimetre on drawings of each section. The relative weight of cortex and medulla was calculated according to the formula:

$$\frac{\text{Weight of thymic cortex (medulla)}}{} = \frac{\text{Thymic weight x Percentage of cortical (medullary) area}}{100}$$

The numbers of lymphocytes, pyknotic and mitotic cells were calculated on an area of 1250 μm^2. The percentage of pyknotic cells and the mitotic index were expressed as:

$$\frac{\text{Number of pyknotic (mitotic) lymphocytes per area}}{\text{Total number of lymphocytes}} \times 100$$

The results, expressed as the mean±SEM, were compared by student's «t» test.

Differences of EB-treated rats to controls are marked in all figures as * $p<0.1$, ** $p<0.01$, *** $p<0.05$ and **** $p<0.001$. Differences between 100 mg and 500 mg EB-treated rats are marked in all figures as o ($p<0.01$), oo ($p < 0.05$) and ooo ($p < 0.001$).

Results

The treatment with both doses of EB induced a statistically significant decrease in the relative thymic weight with respect to values shown by control rats at any tested time (Fig. 1A). The decrease was more pronounced in rats treated with the highest dose, with statistically significant differences to those injected with 100 µg of EB at 7 and 15 days. No recovery of thymus weight was observed at the end of experiment for both used doses.

The pattern of observed changes in thymus weight correlates quite well with that shown by the relative

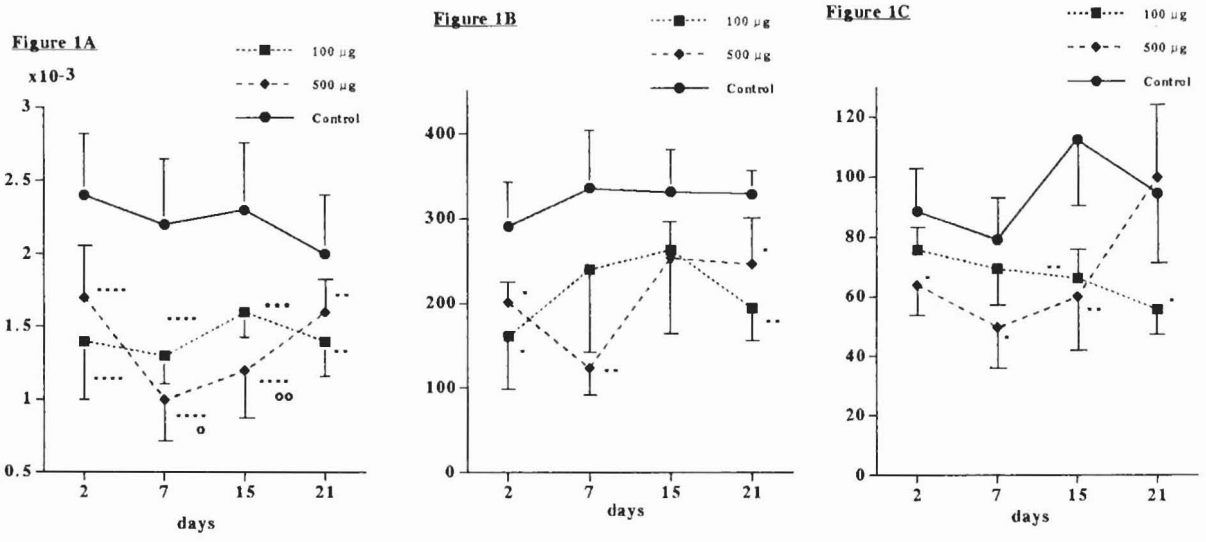

Fig. 1. Relative weight of thymus **(A)**, thymic cortex **(B)** and thymic medulla **(C)** in control, 100 µg, and 500 µg EB-treated rats.

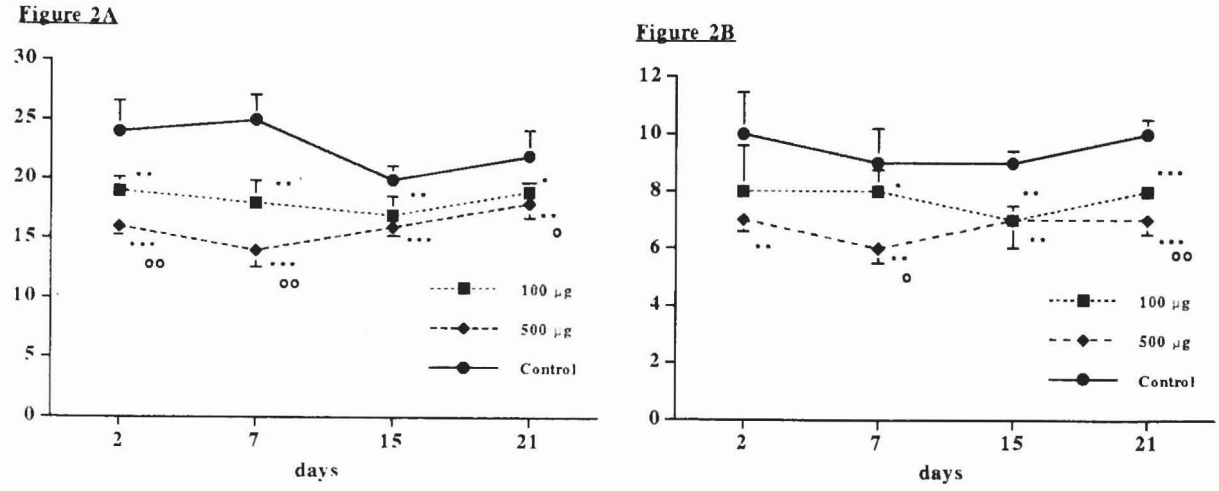

Fig. 2. Frequency (number of lympho-cytes/μm^2) of cortical **(A)** and medullary lympho-cytes **(B)** in control, 100 µg, and 500 µg EB-treated rats.

weights of both thymic cortex (Fig. 1B) and medulla (Fig. 1C). Doses of 100 µg EB induced a sharp fall of the cortical weight 2 days after injection with a gradual recovery at 7 and 15 days stopped by a new fall on day 21 (Fig. 1B). 500 µg EB-treated rats also showed significant decreased cortical weight 2 days after injection, reaching minimal values on day 7 (Fig. 1B). A later gradual increase was sufficient to raise the cortical weight of control rats on day 21 (Fig. 1B). Both doses, but specially the highest one, induced a decrease of the medullary weight 2, 7 and 15 days after treatment (Fig. 1C). On day 21, 500 µg EB-treated rats reached control values, while those receiving the lowest dose maintained a statistically significant decreased medullary weight.

In both cortex (Fig. 2A) and medulla (Fig. 2B) of EB-treated rats, the frequency of lymphocytes decreased significantly in all the days studied. Again the highest dose induced the lowest values on day 7, the number of lymphocytes did not reach control numbers on day 21 and the decrease in number of medullary lymphocytes was specially evident on day 21 (Fig. 2A, B).

In order to analyze the influence of pyknotic cells in the changes observed in the thymic weight of EB-treated rats we calculated the frequency of pyknotic cells per unit area in both cortex and medulla as well as the ratio of both cortical and medullary pyknotic cells to total number of lymphocytes occurring in each thymic area. 7 days after treatment there was an increase in the frequency of cortical pyknotic cells per unit area with statistically significant differences to control values in the 500 µg EB-treated rats but not in those which had received 100 µg of EB (Fig. 3A). Nevertheless, when the changes in the frequency of cortical pyknotic cells were expressed with respect to the total number of

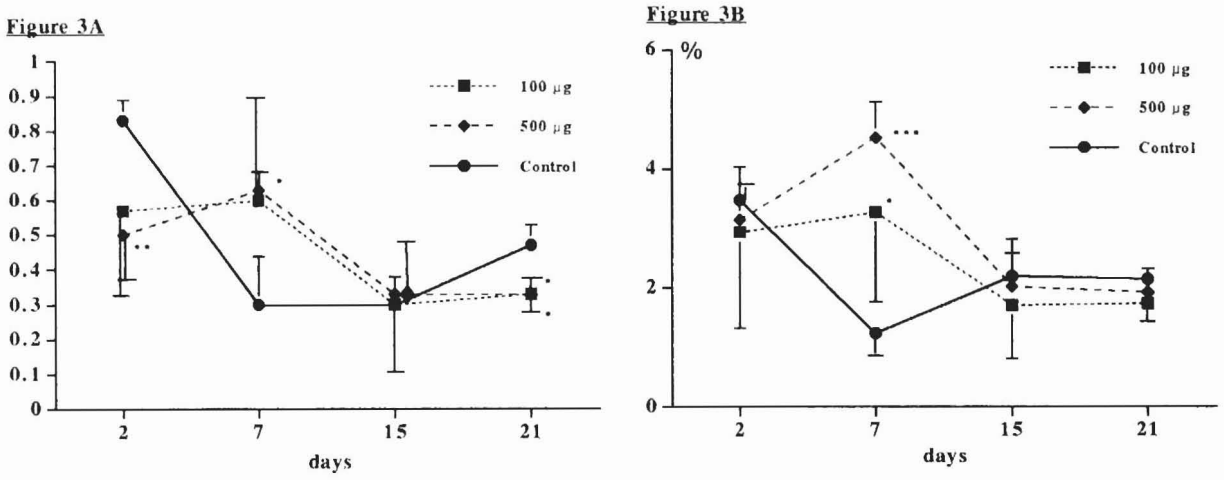

Fig. 3. A. Number of cortical pyknotic cells by unit area in control, 100 µg, and 500 µg EB-treated rats. **B.** Percentage of pyknotic cells with respect to the total number of lymphocytes in the thymic cortex of control, 100 µg, and 500 µg EB-treated rats.

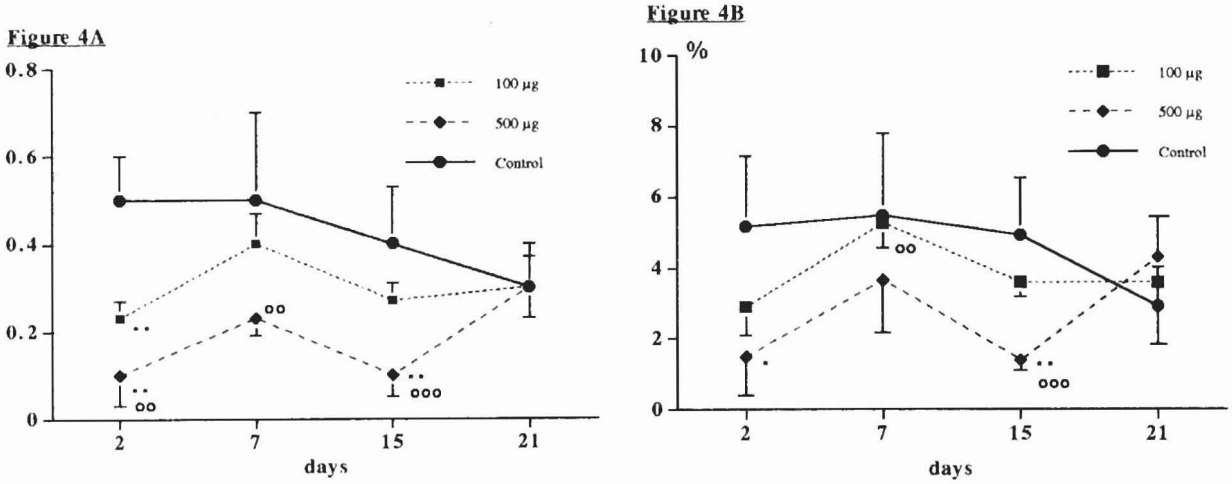

Fig. 4. A. Number of medullary pyknotic cells by unit area in control, 100 µg, and 500 µg EB-treated rats. **B.** Percentage of pyknotic cells with respect to total number of lymphocytes in the thymic medulla of control, 100 µg, and 500 µg EB-treated rats.

lymphocytes, both groups of treated rats showed significantly increased numbers of pyknotic cells 7 days after treatment (Fig. 3B). In the following tested days, the number of cortical pyknotic cells diminished almost to control values, although significant differences were still observed on day 21 when the frequency of pyknotic cells was related to cortical unit area (Fig. 3A). Remarkably, a high number of pyknotic cells occurred in the thymic medulla of control rats (Fig. 4A, B).

On the contrary there was a decrease in the number of medullary pyknotic cells 2, 7 and 15 days after EB-treatment, although statistical differences were found only with the highest dose. Experimental groups reached control values on day 21 (Fig. 4A, B).

With both doses of EB, the frequency of cortical mitotic cells per unit area decreased considerably in all studied days, except 21 days after injection, with respect to control values (Fig. 5A). If the values were expressed with respect to the total number of cortical lymphocytes the low number of cortical mitotic cells persisted in EB-treated rats, although the differences from control values diminished (Fig. 5B). On the other hand, if the number of mitotic lymphocytes observed in the thymic medulla of control rats were considerably lower with respect to those found in the cortex, they raised minimal values in EB-treated rats, specially in the first week after EB administration (Fig. 6A, B).

Discussion

Our morphometrical data demonstrate that estradiol-induced thymic involution, previously observed by numerous authors (Shobon and Jirasatthan, 1974; Screpanti et al., 1982; Kuhl et al., 1983; Luster et al., 1984), correlated well with changes in the lymphoid cell populations, although other studies using the same protocol have also shown profound modifications in the thymic stromal cells, which are not discussed in the present report (Martín-Moreno, 1992). Remarkably, however, these changes affected differentially the thymic lobules and whereas some of them appeared profoundly modified other ones histologically resembled those of control rats.

In agreement with our results, a correlation between high estrogen levels (two days after treatment, the plasma estradiol levels of rats injected with 100 µg of EB increased by three, whereas in those injected with 500 µg of EB the increase was 20 times. EB-treated rats only reached control values 21 days after treatment (data not shown) and thymic involution has been repeatedly

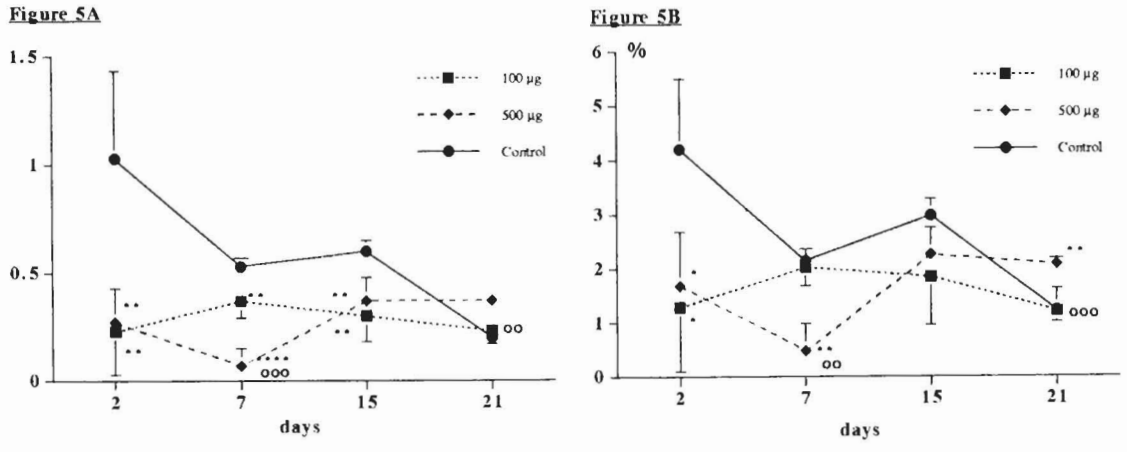

Fig. 5. A. Number of cells in division by unit area in the thymic cortex of control, 100 µg, and 500 µg EB-treated rats. **B.** Percentage of cells in division with respect to the total number of lymphocytes in the thymic cortex of control, 100 µg, and 500 µg EB-treated rats.

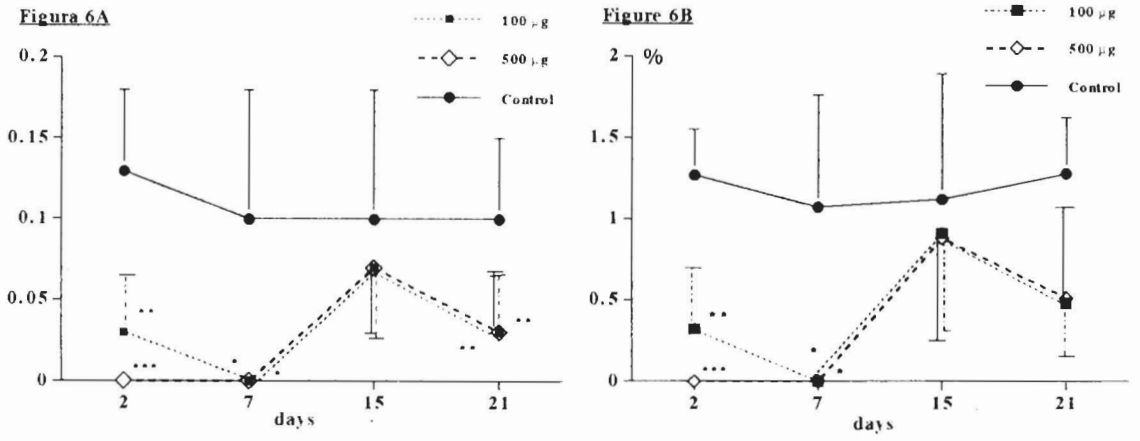

Fig. 6. A. Number of cells in division by unit area in the thymic medulla of control, 100 µg, and 500 µg EB-treated rats. **B.** Percentage of cells in division with respect to the total number of lymphocytes in the thymic medulla of control, 100 µg, and 500 µg EB-treated rats.

claimed in both physiological (Metcalf, 1960; Ito and Hoshino, 1962; Clark and Kendall, 1989; Nakahama et al., 1990) and experimental conditions (Shobon and Jirasatthan, 1974; Screpanti et al., 1982; Kuhl et al., 1983; Luster et al., 1984). Nevertheless, the main causative factor(s) of this involution is controversial. Our results suggest that both cortex and medulla contribute to thymic involution, although the importance of the first one is quantitatively higher. Moreover, although the earliest found changes could be addressed to cortical pyknosis together with a decreased mitotic index and the degeneration of both lymphoid and non-lymphoid cell populations, the mobilization from the thymus is apparently the main factor contributing to both the thymic involution and the lack of recovery observed at the end of experimental period.

Although some authors did not find remarkable changes in the thymic medulla after estrogen treatment (Gulino et al., 1985), others, in agreement with our results, point out that thymic depletion involves both cortex and medulla (Leceta et al., 1988). In this respect, Kuhl et al. (1983) and Shobon and Jirasatthan (1974) have suggested that cortical lymphocytes, as immature, are more susceptible to estrogen effects than medullary ones. Our morphometrical results support that the used highest dose could rapidly affect both immature cortical lymphocytes and more mature medullary lymphoid cells whereas 100 µg EB administration would mainly induce changes in cortical cell populations and only after mobilization of them to the medullary area, this thymic zone could undergo involution.

Furthermore, high levels of sex steroids occurring during the second part of pregnancy (Clark and Kendall, 1989) and after exogenous administration (Kalland et al., 1978; Mysliwska, 1979; Aboussaquira et al., 1991) induce a decrease in the mitotic index of cortical lymphocytes which has been correlated to the low number of lymphoid cells present in the organ. In agreement, our results demonstrate decreased mitotic index in both cortical and medullary lymphocytes, specially after administration of 500 µg of EB. Anyway, since thymic medulla contains mainly mature lymphocytes the observed number of medullary dividing cells is obviously very low in both EB-treated and control rats.

On the other hand, various authors have pointed out the capacity of sex steroids to induce lymphocyte redistribution (Simmons, 1964; Boorman et al., 1980; Gulino et al., 1985). The observed decrease of lymphocyte frequency in both cortex and medulla of EB-treated rats is due to local massive cell degeneration and/or lymphocyte migration from thymus to other lymphoid compartments. However, according to our results only 7 days after EB injection, and especially with the higher dose, there is a remarkable increase in the lymphocyte pyknosis. On the contrary, a cytofluorometric analysis of the affected T-cell subpopulations by EB treatment carried out in parallel to these histological studies has demostrated and important mobilization of both single positive ($CD4^+8^-$ and $CD4^-8^+$) and double positive ($CD4^+8^+$) T-cells from thymus to peripheral lymphoid organs, including spleen and lymph nodes (Casares et al., 1992), a fact previously reported in mouse thymus (Screpanti et al., 1989, 1991).

Nevertheless, although these morphometrical data confirm lymphocyte depletion in both cortex and medulla as a key factor for explaining the estrogen-induced thymic involution, other studies combining ultrastructure, immunohistology and flow cytometry carried out by our group (Casares et al., 1992; Martín-Moreno, 1992) also demonstrate important changes in the thymic non-lymphoid microenvironments after EB-treatment. So, we can conclude that thymic changes induced by estradiol are more profound and complex than previously claimed.

Acknowledgements. This work was supported in part by the Spanish Ministry of Education and Science, grant CICYT PM 890043.

References

Aboussaquira T., Marie C., Brugal G. and Idelman S. (1991). Inhibitory effect of 17ß-estradiol on thymocyte proliferation and metabolic activity in young rats. Thymus 17, 167-180.

Boorman G.A., Luster M.I., Dean J.H. and Wilson R.E. (1980). The effects of adult exposure to diethystilbestrol in the mouse on macrophage function and numbers. J. Reticuloendothel. Soc. 28, 547-560.

Casares F., Moreno A.M. and Zapata A. (1992). Effects of sex steroids on intrathymic T cell differentiation. Abstract 8th International Congress of Immunology. Budapest, Hungary. Abstract 22-7. pp 126.

Clark A.G. and Kendall M.D. (1989). Histological changes in the thymus during pregnancy. Thymus 14, 65-78.

Fonfría J., Barrutia M.G., Garrido E., Ardavín C.F. and Zapata A. (1983). Erythropoiesis in the thymus and spotless starling, *Sturnus unicolor*. Cell Tissue Res. 232, 445-455.

Grossman C.J., Sholiton L.J. and Roselle G. (1982). Estradiol regulation of thymic lymphocyte function in the rat: mediation by serum factors. J. Steroid Biochem. 16, 683-690.

Gulino A., Screpanti I., Torrisi M.R. and Frati L. (1985). Estrogen receptors and estrogen sensitivity of fetal thymocytes are restricted to blast lymphoid cells. Endocrinology 117, 47-54.

Ito T. and Hoshino T. (1962). Histological changes of the mouse thymus during involution and regeneration following administration of hydrocortisone. Z. Zellforsch. 56, 445-464.

Kalland T., Forsberg T.M. and Forsberg J.G. (1978). Effects of estrogen and corticosterone on lymphoid system on neonatal mice. Exp. Mol. Pathol. 28, 76-95.

Kuhl H., Gross M., Schneider M., Weber W., Mehlis W., Stengmuller T.M. and Taubert H.D. (1983). The effects of sex steroids and hormonal contraceptives upon thymus and spleen on intact female rats. Contraception 28, 583-597.

Leceta J., Fernández-Galaz C., Sánchez C., Gomariz R.P., Zapata A. and Tejero A. (1988). Effects of neonatal treatment with estrogens on the development of the thymus in rats. Dev. Comp. Immunol. 12, 375-383.

Leceta J., Garrido E., Torroba M. and Zapata A. (1989). Seasonal

modifications affecting the thymus microenvironment in the turtle *Mauremys caspica*. An ultrastructural study. Cell Tissue Res. 256, 213-219.

Luster M.I., Hayes H.T., Koprach K., Dean J.H., Greenlee W.F. and Boorman G.A. (1984). Estrogen immunosuppression is regulated through estrogenic responses in the thymus. J. Immunol. 133, 110-116.

Martín-Moreno A. (1992). Efectos de los esteroides sexuales sobre el microambiente no linfoide del timo de ratas adultas. Ph.D. Thesis. Complutense University. Madrid, Spain.

Metcalf D. (1960). The structure of the thymus. In: The thymus, recent research in cancer research. Vol. 5. Metcalf D. (ed). Springer-Verlag. Berlin. pp 1-1.

Mysliwska J. (1979). Reactivity to estradiol of thymic cells from female mice before and after puberty. Endocrinology 73, 61-65.

Nakahama M., Mohri N., Mori S., Shindo G., Yokoi Y. and Macchinami R. (1990). Immunohistochemical and histochemical studies of the human thymus with special emphasis on age-related changed in medullary epithelial and dendritic cells. Virchows Arch. (B) 58, 245-251.

Screpanti I., Gulino A. and Pasqualini J.R. (1982). The fetal thymus of guinea pig as an estrogen target organ. Endocrinology 11, 1552-1560.

Screpanti I., Morrono S., Meco D., Santoni A., Gulino A., Paolini R., Crisanti A., Mathieson B. and Frati L. (1989). Steroid sensitivity of thymocyte subpopulations during intrathymic differentiation. Effects of 17ß-estradiol and dexamethasone on subsets expressing T cell antigen receptor of IL-2 receptor. J. Immunol. 142, 3378-3383.

Screpanti I., Meco D., Morrone S., Gulino A., Mathieson B. and Frati L. (1991). *In vivo* modulation of the distribution of thymocyte subsets: Effects of estrogen on the expression of different T cell receptor Vb gene families in $CD4^-$, $CD8^-$ thymocytes. Cell Immunol. 134, 414-426.

Shobon P. and Jirasatthan C. (1974). Effect of sex hormones on the thymus and lymphoid tissue of ovariectomized rats. Acta Anat. 89, 211-225.

Simmons P.V. (1964). Film abstract of discussion in leukopoiesis in health and disease. Ann. N.Y. Acad. Sci. 133, 948-953.

Zapata A., Garrido E., Leceta J. and Gomariz R.P. (1983). Relationships between neuroendocrine and immune system in anphibians and reptiles. Dev. Comp. Immunol. 7, 771-774.

Accepted December 7, 1993