Virchows Archiv A Pathol Anat (1992) 421:505–511

Virchows Archiv A
Pathological Anatomy and Histopathology

Oestrogen retards the development of spontaneous thymomas in BUF/Mna rats

Taichi Ezaki[1], Hirohiko Fujii[1], Kenjiro Matsuno[1], Ryuichi Kawatsu[2], and Masahiko Kotani[3]

Departments of [1] Anatomy and [2] Surgery, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860, Japan
[3] Kyoto College of Medical Technology, 1-3 Imakita, Koyama-higashi-machi, Sonobe-cho, Funai-gun, Kyoto 622, Japan

Received June 9, 1992 / Received after revision September 2, 1992 / Accepted September 4, 1992

Summary. BUF/Mna rats develop spontaneous thymomas with nearly 100% incidence in both sexes. While the thymomas in males develop from around 9 months of age, those in females start from 13–15 months of age. To clarify the mechanism of the delay of thymomagenesis in females, the effect of sex hormones on the development of thymomas was examined after either gonadectomy or oestrogen treatment. Prepubertal ovariectomy accelerated the thymoma development in females, whereas orchiectomy did not affect it. An intraperitoneal injection of oestriol (20 mg) into males at 2 months of age remarkably diminished the thymic weight to about one-tenth of age-matched controls at 16 months of age. These results suggest that oestrogen can actually retard the onset of thymoma in spite of genetic control of its incidence. However, oestrogen did not cause thymic involution when it was injected into rats over 9 months of age. Immunohistochemically, there seemed to be no distinct difference in distribution of oestrogen-receptor-bearing epithelial cells between thymomas and 2- to 3-month-old thymuses. The oestrogen sensitivity of the thymus might be destined to be lost, as the thymic epithelial cells start neoplastic changes with the impairment of oestrogen-receptor function.

Key words: Inbred BUF/Mna rats – Thymoma – Oestrogens – Oestrogen receptor

Introduction

Thymoma is defined as a neoplasm of epithelial origin with various proportions of lymphocytes (Rosai and Levine 1976). The tumour is of particular interest because of its close association with various immunological disorders, including certain types of autoimmune disease. In human thymomas, there has been no evidence of any preferences for a particular sex (Rosai and Levine 1976; Rosenow and Hurley 1984). Although spontaneous thymomas are fairly rare in laboratory animals, several investigators have reported a sex bias in the incidence of rat thymomas. In most of the strains reported so far, female rats have revealed a higher incidence of the tumour than males (Dunning and Curtis 1946; Maekawa and Odashima 1975; Murray et al. 1985). Indeed gonadectomy raised the rate of thymoma incidence in WAB rats of both sexes (Hinsull and Bellamy 1977), but no explanation for its mechanism has been proposed.

BUF/Mna rats develop spontaneous thymomas and the tumours closely resemble human thymomas (Yamada et al. 1973). Their incidence is regulated by an autosomal dominant gene, *Tsr-1* (Matsuyama et al. 1986, 1988). Pathologically, the thymomas are benign in nature and classified as the lymphocyte-predominance type with remarkable increase of hyperplastic lymphocytes (Yamada et al. 1973; Ezaki et al. 1990). This strain of rats has provided a unique model to study thymic microenvironment for T-cell differentiation (Ezaki et al. 1991a, b). Recently, we examined the onset time of the thymomas in both sexes and found that there was a distinct delay of the thymomagenesis in females. In the present study, we have investigated the possible effect of sex hormones on the development of thymomas to clarify the mechanism of the delay of thymomagenesis in female BUF/Mna rats by gonadectomy and oestrogen treatment.

Materials and methods

Inbred Buffalo/Mna (BUF/Mna) rats were bred by sib-mating in our rat colony at the Laboratory Animal Centre for Experimental Research, Kumamoto University Medical School. They were maintained in an air-filtered clean room and fed with sterilized standard laboratory chow and water ad libitum. The rats were killed at various ages (up to 24 months) by cervical dislocation under ether anaesthesia. A total of 128 rats (78 males, 50 females) were used for the study of the development of thymomas. In other experiments, 3–8 rats per group were analysed as described below in detail.

Correspondence to: T. Ezaki

According to Yamada et al. (1973), a thymus weighing more than 2.0 g was expediently regarded as thymoma. Young (up to 3- to 4-month-old) thymuses before a temporal physiological involution never grow to 2.0 g, whereas most of those weighing more than 2.0 g after the involution period were accompanied by pathological changes of thymoma (Ezaki et al. 1990).

Gonadectomy was performed at 1 month of age under ether anaesthesia. In male rats ($n=4$), both testes and epididymis were excised, after a ligation of the vas deferens and spermatic vessels, through a mid-line incision of scrotal skin. In females ($n=4$), ovaries with adjacent segments of the uterine horns were removed through bilateral small incisions of the dorsal skin. The skin incisions were closed by wound clips (Clay-Adamms, Parsippany, N.J., USA). Control rats (4 males, 3 females) were sham-gonadectomized in the same way. The animals were sacrificed at 16 months after birth.

For short-term experiments, male rats at the ages of 1.5 months ($n=6$), 6 months ($n=4$), 9 months ($n=4$), 12 months ($n=3$), 20 months ($n=8$) and 22 months ($n=4$) were intraperitoneally injected (i.p.) with 30 mg of oestriol (E_3) in 3 ml of aqueous suspension (Estriel: Mochida Pharmaceutical Co., Tokyo, Japan) per 100 g body weight. Control male rats at the corresponding ages ($n=4$, 6, 3, 5, 8 and 8, respectively) were given the same volume of solvent (Mochida) without E_3 in the same manner as previously described (Kawatsu et al. 1989). Rats were killed 15 days after the E_3 administration. For long-term experiments, 5 male rats of 2 months of age were injected i.p. with 20 mg E_3 and maintained until 16 months of age. Controls rats from the same littermates (3 males, 3 females) were given the same volume of solvent without E_3 in the same manner. As one of the oestrogenic hormones, we have employed E_3 in this series of experiments because E_3 has broad and quite strong actions on various cells and tissues instead of its relatively weak action on the reproductive organs as primary targets (Kawatsu et al. 1989; Kotani 1990).

To induce and detect oestrogen receptor (ER) in thymic tissue sections with maximal efficiency, oestrogen-primed male rats were prepared by receiving daily i.p. injections of E_3 (1 mg) for 4 days according to the method of Kawashima et al. (1991) with modification. One day after the last injection, thymuses were removed and frozen in liquid nitrogen for the following ER immunostaining.

Mouse monoclonal antibodies (mAbs) against rat lymphoid cells – OX19 (anti-CD5) and OX8 (anti-CD8) (Sera-Lab., Sussex, UK); R73 (anti-α/β heterodimeric T-cell antigen receptor) (Serotec, Oxford, UK) – were used to determine the phenotypes and distribution of the T-cell populations. A mouse mAb (ER-D5) raised against a human cytosolic ER component (King et al. 1985) was obtained from Amersham International (Amersham, UK). ER-D5 can precipitate receptor-bound oestradiol in a hormone-specific manner and recognizes an epitope on a cytoplasmic 29000 molecular weight (mol.wt.) protein (Coffer et al. 1985; King et al. 1985). The cross-reactivity of ER-D5 with rat ER in the thymus and uterus has also been confirmed biochemically and immunohistochemically by Kawashima et al. (1991). Although ER-D5 appears to react with a receptor-related antigen that is different from the recent concept of the ER (King and Greene 1984) and the physiological relevance of the 29000 mol.wt. antigen remains to be elucidated, we used the mAb in this work as a pilot study to predict the presence of ER-bearing cells in the rat thymus by demonstrating the ER-related antigen.

Three to five thymuses in an individual sample group were examined. Thymuses from 2- to 3-month-old male rats and 16- to 19-month-old male rats were fixed in Carnoy's fluid, dehydrated in ethanol and embedded in paraffin wax. Serial 4-μm-thick sections were made and stained with methyl green-pyronine or haematoxylin and eosin.

For immunohistochemistry, thymuses were freshly frozen in liquid nitrogen and 6-μm-thick cryosections of these tissues were made. In immunostainings for ER-bearing cells, the cryosections were fixed in 0.2% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS) at 4° C for 5–10 min. The indirect immunoenzymatic method was employed as described in detail previously (Matsuno et al. 1989). Briefly, sections were incubated with various mAbs for 60 min at room temperature and washed in PBS. A horseradish peroxidase (HRP)-conjugated rabbit antibody to mouse immunoglobulins (1:200; DAKO, Copenhagen, Denmark) was used as a secondary antibody. The HRP reaction was developed in either 3′-diaminobenzidine hydrochloride (Dojin Chemicals, Kumamoto, Japan) solution or 4-chloro-1-naphthol (Sigma, St. Louis, Mo., USA) solution. All negative controls stained with either normal mouse serum or unrelated mouse mAbs instead of specific antibodies showed no or only negligible level of background stain.

Body weight and thymic weight from at least 3 (usually 4–6) animals in individual sample groups were measured and analysed. Statistical significance of differences in mean values was assessed using Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

Results

The development of thymomas in both sexes (78 males, 50 females) was compared by measuring thymus weight and body weight at various ages. The results were expressed as percent thymus weight per body weight as shown in Fig. 1. The values of both sexes showed no significant difference until 6–7 months of age. In male rats, however, thymuses reincreased rapidly at about 9 months of age (the onset of the tumours) after a temporal physiological involution. In females, the temporal thymic involution lasted for a longer period and thymuses started reincreasing their weight about 13–15 months after birth. The delay of thymomagenesis in females was usually 4–6 months. The incidence of the thymoma was 98% in males ($n=44$) at above 12 months of age and 88% in females ($n=24$) at above 18 months of age.

Histologically, there is no significant sex difference in the thymic structure until 6–7 months of age. After this period, the following structural changes occur in both sexes, but more slowly in females than in males. Thymuses from young (2- to 3-month-old) rats of both sexes have normal structure of thymic lobules with well-

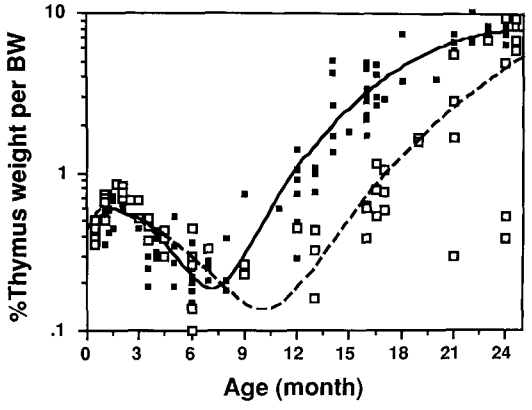

Fig. 1. Changes in thymus weight with aging. The values are expressed as percent thymus weight per body weight (*BW*) for comparison. Each *point* represents individual animals (■, males; □, females) at various ages. *Lines* (*continuous*, males; *broken*, females) were drawn for the best fit from each point by the polynomial regression curve analysis (*continuous line*, males; $r^2=0.90$; *broken line*, females; $r^2=0.73$)

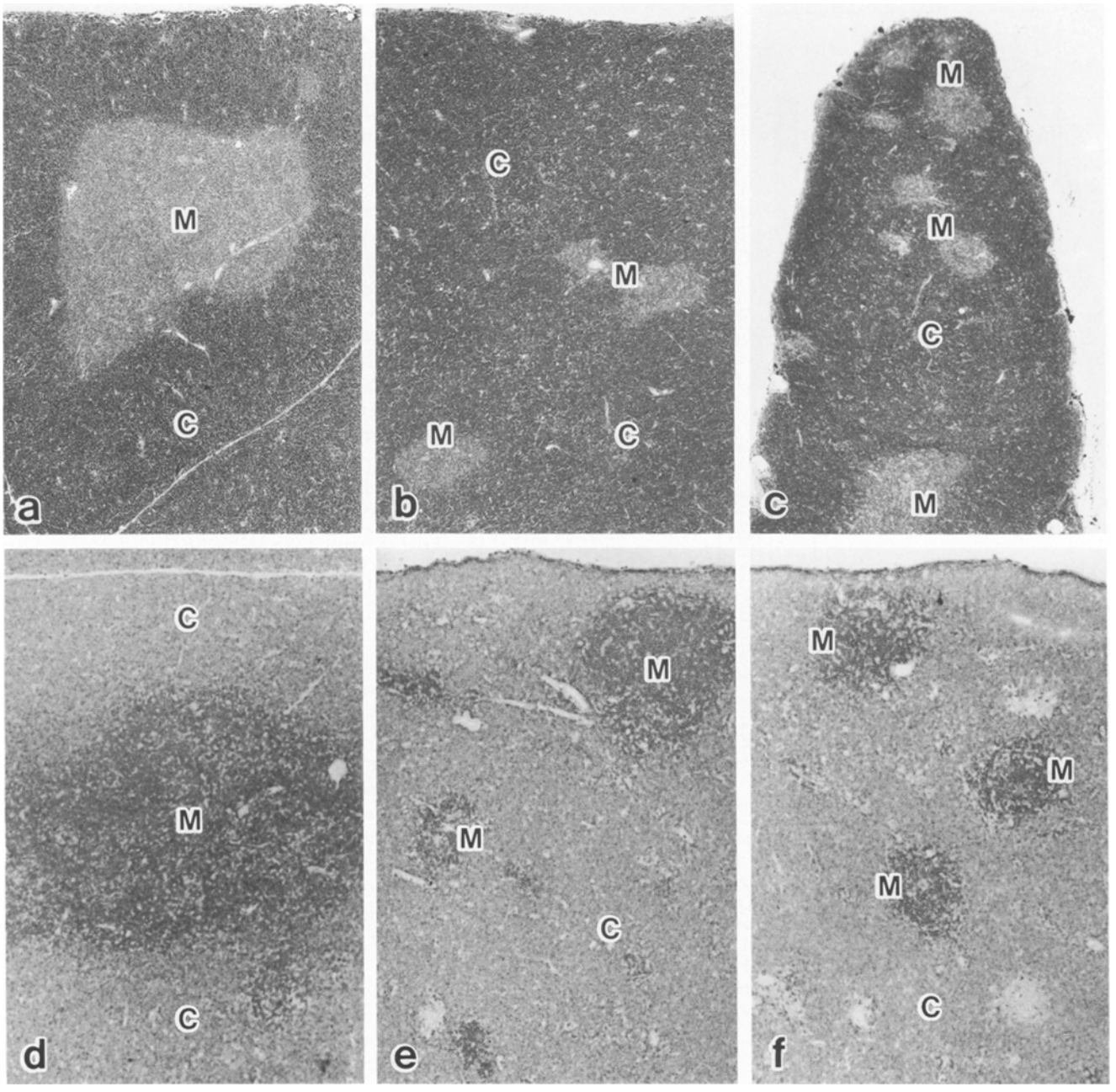

Fig. 2. Microscopical structures of typical young (2-month-old) thymuses (**a, d**), 16-month-old thymomas (**b, e**) and E_3-treated 16-month-old thymuses (**c, f**). Note the remarkable increase in cortical areas (*C*) and decrease in medullary areas (*M*) in the thymomas. Medullary thymocytes express more T-cell antigen receptor than cortical cells. **a–c** Methyl green-pyronine stain, ×42; **d–f** immunoperoxidase staining with R73 mAb, ×55

developed medulla (Fig. 2a). Gradually with age, the medulla becomes narrower and more segmented due to the cortical enlargement. In typical thymomas of 16-month-old male rats, the cortical areas occupy most parts of the expanding thymic lobules and the medullary areas become segmented like small islets (Fig. 2b). The distribution patterns of T-cells in the thymus were also examined by the immunostainings for various T-cell markers. R73 mAb, against T-cell antigen receptor, recognizes medullary thymocytes much more intensely than cortical thymocytes (Hunig et al. 1989). Immunostaining with R73 reveals that the distributions of T-cells both in young thymuses (Fig. 2d) and in thymomas (Fig. 2e) correspond well to their appearance in paraffin sections (Fig. 2a and b, respectively) in terms of the cortex-medulla ratio. The immunostainings for other T-cell markers (CD5 and CD8) also show the cortex-predominant appearance (data not shown).

In order to examine the roles of sex hormones in the development of thymomas, we employed two approaches; the withdrawal and the exhibition of sex hormones. Firstly, prepubertal rats of both sexes were gona-

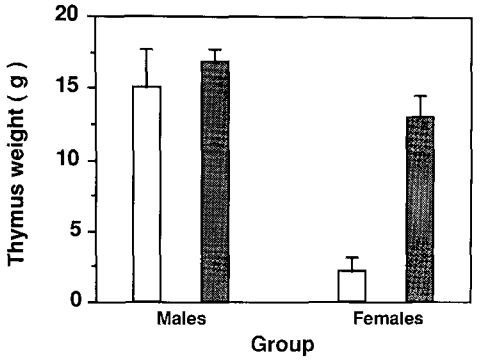

Fig. 3. Effect of gonadectomy on thymoma development. Rats were either gonadectomized (4 males, 4 females: *shaded column*) or sham-operated (4 males, 3 females: *open column*) at 1 month of age and their thymus weight was measured when they were 16 months old

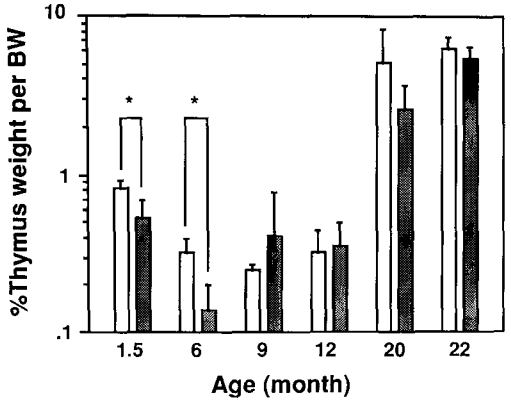

Fig. 4. Oestrogen sensitivity of thymuses at various ages. Male rats of various ages were injected i.p. either with 30 mg E_3/100 g body weight (*shaded column*) or with same volume of solvent (*open column*). Thymus weight was measured 15 days after the hormone treatment and the values were expressed as percent thymus weight per body weight (*BW*) for comparison. Each column represents means of 3–8 rats and *bars* represent standard deviations of the means. Differences in the values between E_3-treated and control rats were statistically significant at 1.5 and 6 months of age (*)

dectomized at 1 month of age and the effects of the gonadectomy on the development of thymomas were examined at 16 months of age (Fig. 3). At this age, females had just started developing thymomas, whereas almost all males had already developed huge thymomas. There was, however, a dramatic increase of thymus weight in ovariectomized females and the weight reached the levels of male rats. No significant effect of orchiectomy was seen in males. Histologically, there is no significant difference in general structures of the thymoma between the ovariectomized females, sham-operated control males and orchiectomized males (data not shown). The body weights of ovariectomized females (330 ± 5.3 g) and orchiectomized males (358 ± 13.9 g) were almost in the same range, in contrast with their sham-operated controls: females (255 ± 36.0 g) and males (477 ± 26.3 g), respectively. Secondly, the effect of oestrogen imposition on the development of thymomas was examined in the long-term experiments. Two-month-old male rats were injected i.p. with 20 mg E_3 and the development of thymomas was examined at 16 months of age (Table 1). The single administration of E_3 strongly suppressed the enlargement of thymuses and the thymuses of oestrogenized rats remained small, i.e. even smaller than those of non-treated 16-month-old females. Histologically, general thymic structures and the distribution of T-cells in the thymuses from E_3-treated rats (Fig. 2c, f) resemble the typical thymomas (see Fig. 2b and e, respectively) except for their size. This indicates that E_3 could not completely suppress the neoplastic changes in the thymus but affected the size of the cortical areas.

The sensitivity of thymuses to oestrogen at various ages was also investigated. Male rats at various ages were injected i.p. with 30 mg E_3/100 g body weight and were killed 15 days after the E_3 administration. These short-term experiments enabled us to estimate the possible effect of E_3 with acute changes, if any, in the thymic weight. The oestrogen was only effective on relatively young (up to 6 months of age) thymuses and showed no significant effect after 9 months of age (Fig. 4).

Finally, we investigated the histological distribution of ER-bearing cells to see if there is any change in the level of ER-related molecule in the thymus. Cryosections of thymuses from 3-month-old and 19-month-old rats were immunostained with ER-D5 mAb. In 3-month-old thymuses, ER-bearing cells are localized mostly in the cortex (Fig. 5a). In 19-month-old thymomas, ER-bearing cells are seen throughout the enlarged cortex (Fig. 5b). They are large cells with dendritic processes containing round or oval nuclei. Some of them form honeycomb-like or ring-like cellular complexes like thymic nurse cells (Fig. 5c). There is no distinct difference in the cellular density and staining intensity comparing with those in 3-month-old thymuses.

Table 1. Effect of oestrogen on the development of thymomas

Sex	Oestriol treatment[a]	Body weight (g)	Thymus weight (g)	Thymus wt./body wt.
Female	–	264 ± 2.3	2.72 ± 0.29	1.0 ± 0.11
Male	–	423 ± 30.6	17.21 ± 3.87	4.1 ± 1.11
Male	+	434 ± 32.4	1.66 ± 0.85	0.4 ± 0.18

[a] Oestriol (20 mg) was injected i.p. when rats were 2 months old; the development of thymomas in these rats was examined at 16 months of age

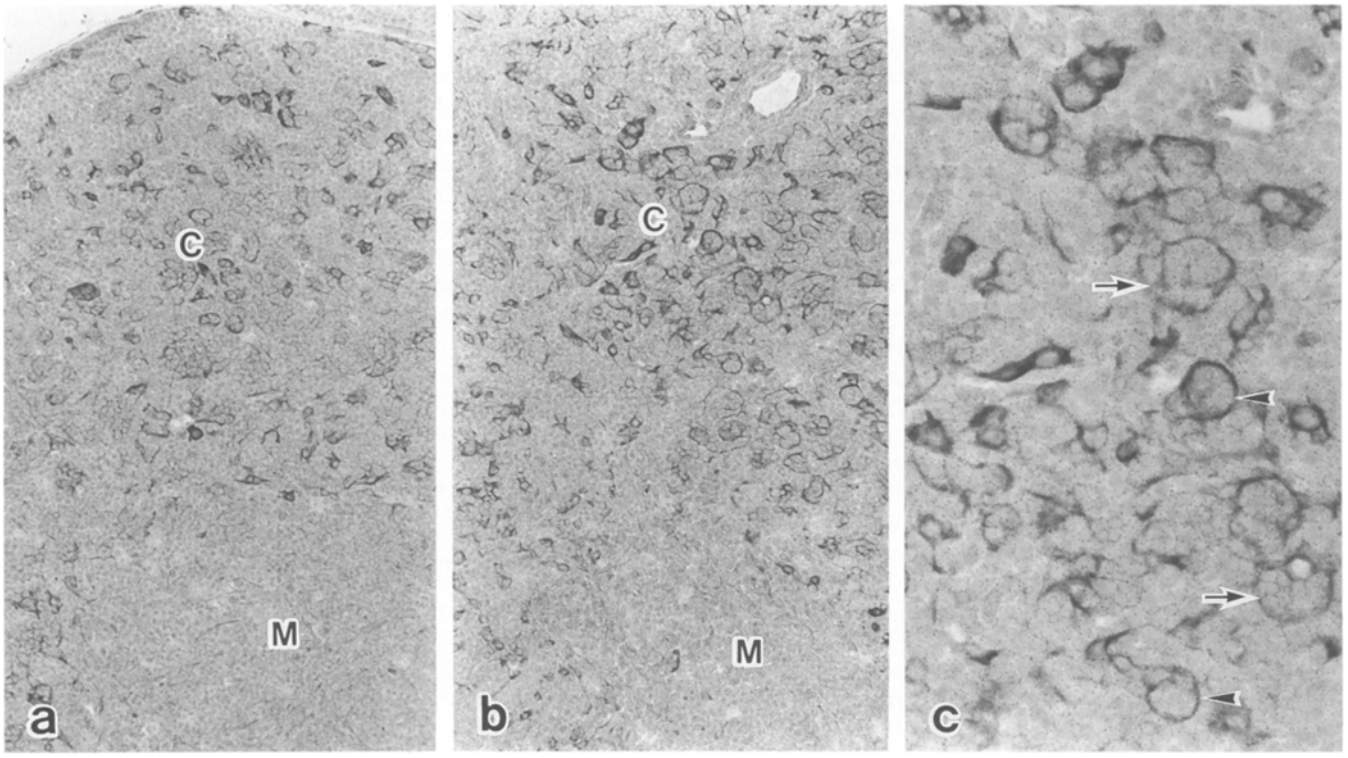

Fig. 5a–c. Immunoperoxidase staining of oestrogen receptor (ER)-related antigen in the thymus. Cryosections of 3-month-old thymuses (**a**) and 19-month-old thymomas (**b**, **c**) were immunostained with ER-D5 mAb. Note that most ER-bearing cells of both ages are localized in the cortex (*C*), whereas far fewer are in the medulla (*M*). Some of the ER-bearing cells form honeycomb-like (*arrows*) or ring-like (*arrowheads*) cellular complexes. **a**, **b** ×135; **c** ×340

Discussion

The most important and intriguing finding in this study is that oestrogen retarded tumour development, even though it could not completely suppress the neoplastic changes in the thymus. This was shown under both physiological and pharmacological conditions. Prepubertal ovariectomy caused a remarkable increase of thymic weight in females at 16 months of age, whereas orchiectomy did not influence tumour development. Moreover, the administration of an exogenous oestrogen inhibited the ordinary development of thymomas in male BUF/Mna rats. These results strongly suggest that oestrogen, if not exclusively, can actually inhibit or retard the development of thymomas, whereas androgenic hormones may not relate directly to the tumour development. It is uncertain, however, whether a similar mechanism is involved in the sex bias of other rat thymoma models (Dunning and Curtis 1946; Maekawa and Odashima 1975; Hinsull and Bellamy 1977; Murray et al. 1985). Based on the concept of the hypothalamic-pituitary-gonadal-thymic axis (Grossman 1985), Hirokawa et al. (1990) reported that hypophysectomy prevented the thymoma development in male BUF/Mna rats. However, the hypoplastic thymuses in these hypophysectomized rats could be explained by changes in the level of any hormones, or simply by the severe malnutrition due to the multi-organ dysfunction. In the present study, neither gonadectomy nor E_3 treatment caused any signs of malnutrition such as body weight loss.

The actual mechanism of the suppressive effect of oestrogen on thymoma development has yet to be established. It is assumed that oestrogen regulates a normal cellular differentiation process in the thymus through its receptor (Grossman 1989). ERs in the thymus have been detected and localized predominantly in the reticuloepithelial cells (Stumpf and Sar 1976; Grossman et al. 1979; Seiki et al. 1988). It has also been reported that sex steroids regulate the biosynthesis and secretion of several thymic hormones, such as thymosin and thymulin (Grossman 1985). Oestrogens depress thymosin release from the thymus (Savino et al. 1988), thus decreasing levels of oestrogen, like ovariectomy, may facilitate thymosin release and then increase the size of thymus. Kawashima et al. (1991) have demonstrated the simultaneous presence of both ERs and thymulin in some thymic epithelial cells, proposing a tentative link between oestrogen and thymic factors. Toyokawa et al. (1987) reported that thymic epithelial cells from BUF/Mna rats secreted a novel factor that stimulated the proliferation of immature cortical thymocytes. Oestrogen might also have affected the production of this factor in BUF/Mna rats. In any case, the effect of oestrogen on the thymus may be primarily mediated by the altered epithelial components rather than the massive thymocytolysis which is seen in case of glucocorticoid treatment (Dougherty

1952; Kotani 1990). However, since oestrogens also strongly activate the macrophage population (Vernon-Roberts 1969; Slijivic and Warr 1973; Ezaki et al. 1982), macrophages might play an important role in regulating the proliferation of epithelial cells. Alternatively, an enhanced clearance of thymocytes by macrophage populations in the thymus might have resulted in thymic involution. These possibilities should be elucidated further.

The oestrogen sensitivity of the normal thymus seems to differ among species and ages (Mysliwska 1979; Kendall 1988; Hart 1990). In BUF/Mna thymuses, the administration of oestrogen was only effective when the hormone was given in young animals (less than 6 months of age). Thus, the presence of oestrogen at relatively young ages may be critical in this tumour model, and the loss of oestrogen sensitivity in the older thymuses was not merely due to the physiological decline of oestrogen level with aging. Since the thymomagenesis starts as early as 9 months of age in male rats (Ezaki et al. 1990), the loss of oestrogen sensitivity may be closely related to the onset of the thymoma or neoplastic changes of the epithelial cells. In order to answer the question of whether the loss of oestrogen sensitivity was due to the reduction of ER-positive cells, we examined the tissue distribution of ER-bearing cells in the thymus using a mAb to ER (ER-D5). There was no distinct difference in the histological distribution of ER-bearing cells between 3-month-old thymuses and 19-month-old thymomas. The ER-bearing cells showed the characteristic features for those of epithelial cell component of the thymus. Some of them looked like the typical thymic nurse cells (Ezaki et al. 1991a, b), though their immunobiological significance remained to be clarified. Kawashima et al. (1991) have confirmed that the ER-bearing cells recognized by ER-D5 mAb are also keratin-positive and FTS(facteur thymique serique or thymulin)-positive, suggesting they are in fact epithelial cells. Thus, the ER of the epithelial cells in thymomas might be functionally impaired with respect to the involution effect of oestrogens, as discussed above. In other words, it might be possible that the neoplastic transformation of epithelial cells brings some functional changes in the process where oestrogens act through their receptors, such as "receptor processing" (Edwards et al. 1979) or "receptor activation" (Giambiagi and Pasqualini 1989). Recently, Hirokawa et al. (1990) demonstrated that some phenotypic changes in thymic epithelial cells were important in the development of thymomas. Particularly, the enhanced expression of epidermal growth factor (EGF) and thymosins in the thymus may play important roles in the proliferation of epithelial cells and lymphocytes, respectively. The regulation of EGF receptor by oestrogen in the rat uterus has been reported (Mukku and Stancel 1985). A significant increase in interleukin-1a mRNA has been also demonstrated in the intact thymus after oestrogen treatment (Screpanti et al. 1991). As an ideal biological marker for oestrogen sensitivity, it would be interesting to examine the intrathymic levels of these various functional molecules that are regulated by oestrogens.

In BUF/Mna rats, it has also been reported that there is a clear preponderance of females over males in the grade of glomerular sclerotic lesions with proteinuria, though its development is controlled by two autosomal recessive genes (Matsuyama et al. 1990). Furthermore, an exacerbated weakness of hind-limb muscles (Kato and Watanabe 1982) occurs predominantly in males, whereas a preferential incidence of cataract is seen in females of this rats, though its incidence is relatively low (unpublished observations). It is of great interest to know whether sex hormones play any important roles in the pathogenesis of these diseases in this animal model.

Acknowledgements. The authors are grateful to Professor K. Seiki and his colleagues (Tokai University Medical School, Kanagawa, Japan) for their invaluable advice and criticism relating to oestrogen receptor analyses; to Professor Y. Uehara (Kumamoto University Medical School) for his encouragement and support: and to Mrs. E. Kinoshita for her technical assistance. This work was supported by the Okukubo Memorial Fund for Medical Research at the Kumamoto University Medical School.

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