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Involution of the rat thymus in experimentally induced hypothyroidism

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Abstract. The thymus, as part of the immune-neuroendocrine axis, is greatly influenced by factors from most endocrine glands, especially the thyroid. Antithyroid drugs (carbimazole and methimazole) were used to induce hypothyroidism in rats. Histological and ultrastructural examination of the thymus showed progressive thymic involution after 4 weeks of drug treatment to the end of observations (7 weeks). The involution was characterised by increased thymocyte apoptosis and thymocyte phagocytosis by macrophages. This resulted in thymocyte depopulation, increases in numbers of interdigitating cells, alterations to mainly subcapsular and medullary epithelial cells, an apparent increase of mast cells and collagen in the capsule and septa, and increased numbers of B cells and plasma cells. Lymphoid cells immuno-reactive with MRC OX12 (which detects B cells) were observed within blood vessel walls, suggesting that they may have been moving in and out of the thymus. The administration of drugs causing hypothyroidism, therefore, also caused marked involution of the thymus.

Key words: Thymus – Hypothyroidism – Plasma cells – Anti-thyroid drugs – Rat (Wistar, CSE)

Introduction

Many studies have shown that hormones influence thymic structure. In particular, the adrenocortical and related steroid hormones induce marked thymic involution (Dougherty and White 1945; Ito and Hoshino 1962; Cowan and Sorenson 1964; Haelst 1967; Dourov 1974). This involution is due, at least partially, to massive cortical thymocyte death and extensive phagocytosis caused by steroid-induced apoptosis of immature cortical thymocytes (see Kendall 1990). In hydrocortisone-induced thymic involution, thymocyte destruction typically begins with altered nuclear morphology 4–8 h after an initial injection and involution is maximal after 2 days when injections are given for 2 consecutive days. Although thymic involution occurs after thyroidectomy and/or the administration of anti-thyroid factors (Pacini et al. 1983), the histological basis of the involution has not been studied.

Today, it is well accepted that the thymus is part of a complex immuno-neuroendocrine system (Besedovsky and Sorkin 1981; Millington and Buckingham 1992) and that thymic hormones secreted by epithelial cells of the subcapsular cortex and medulla play an important role (see Kendall 1991). Within the thymus these hormones induce T cell markers and affect the maturation of thymocytes thus potentially altering the peripheral T cell subset ratios (Kendall et al. 1992). Several neuroendocrine factors potentiate the release of thymic hormones (Millington and Buckingham 1992). Fabris et al. (1986) have shown that thymic hormone activity is modulated by thyroid hormones, and Pacini et al. (1983) presented indirect evidence that the outflow of T cells from the thymus is reduced in thyroid dysfunction. Thus there is an interrelationship between the thyroid gland and the thymus.

The present work documents ultrastructural and immuno-histochemical changes resulting from the induction of hypothyroidism in adult male rats.

Materials and methods

Two experiments were conducted. A preliminary experiment in Egypt used 20 male Wistar rats (15 experimental and 5 control) with an average body weight of 250 g. Each rat was orally dosed every other day with 12 mg/kg body weight of the antithyroid drug, carbimazole (Neo-mercazole, Nicholas Laboratories, Welwyn Garden City, UK) dissolved in 0.5 ml of 0.9 M saline, or with saline alone (controls). All rats were sacrificed with ether overdose 4 weeks after drug administration commenced; thymus and thyroid were prepared for conventional light microscopy and thymus for enzyme histochemistry.

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The experiment conducted in the UK used 35 (13 experimental and 22 control) adult male CSE rats weighing 240–260 g. Every other day from time 0, these animals received intraperitoneal injections of 12 mg/kg body weight methimazole (the biologically active form of carbimazole, kindly supplied by Nicholas Laboratories, Welwyn Garden City, UK), dissolved in 0.5 ml of 0.9 M saline or saline alone (controls). Rats were sacrificed after 4 weeks (3 control and 7 experimental), 6 weeks (6 control and 10 experimental) and 7 weeks (4 control and 5 experimental) for light- and electron-microscopic (LM and EM) studies.

Rats were anaesthetised with pentobarbitone (40 mg/kg body weight) and blood withdrawn from the tail vein into microhaematocrit tubes for the estimation of serum tetraiodothyronine (T_4) by use of T_4 RIA kits (Amersham Diagnostics, Little Chalfont, UK). Nineteen of these rats were then fixed by perfusion (0.1 M cacodylate-buffered 2.7% glutaraldehyde and 0.8% paraformaldehyde, pH 7.4) for LM and EM studies. The thyroid and thymus were excised, the thymus weighed and small (1 mm^3) samples of the perfused thymus were immersed in the same fixative for a further 2–4 h at 4°C, then embedded in Epon 812. Semithin sections stained with Methylene blue/Azur II (Richardson et al. 1960) were used to quantitate the mean distance between adjacent cortical epithelial nuclei (see Kendall 1986). Briefly, five regions were selected at random in the mid-cortex on 2 non-sequential sections from each of 12 rats. Within each of these regions, the distance from one type 2 or type 3 epithelial cell nucleus to the next nearest was measured (at a magnification of × 400) to give 5 values for inter-epithelial cell distances. Ultrathin (silver/gold) sections were cut and stained with uranyl acetate and lead citrate (Reynolds 1963).

Larger pieces of thymus and thyroid were fixed in formol acetic alcohol for paraffin embedding and sections cut at 4 μm. These were stained with haematoxylin and eosin, stains for reticular fibres (Gordon and Sweets 1936) and for mast cells (Csaba 1969). The height of the epithelial cells in 10 thyroid follicles were measured (at × 160) in 3 control and 3 experimental rats sampled after 4 weeks of treatment. For estimating the mean ± SEM) number of mitotic figures per mm^2 within the thymic cortex, a squared graticule (covering 0.62 mm^2) was positioned with one edge just inside the capsule at 5 different parts of the cortex on 2 sections from each gland, and the mitotic figures in these areas were counted (at × 160).

Counts of thymocytes were performed on whole thymuses dissected from 3 control and 3 methimazole-injected rats killed after 6 weeks. The tissues were dissociated in Hanks' balanced salt solution using a fine meshed stainless steel sieve. The viable cells (trypan blue exclusion test) recovered were counted in a Thoma haemocytometer.

Frozen tissue sections (5 or 8 μm thick) prepared from 9 rat thymuses (UK experiment), frozen in isopentane cooled in liquid nitrogen were used for immuno- or acid phosphatase-histochemistry (Gomori 1941). The monoclonal antibodies used (given by the MRC Cellular Immunology Unit, Oxford, UK) were: W3/13 for all T cells and polymorphs (Dallman et al. 1984); MRC OX35 for rat CD4 antigen (Jefferis et al. 1985); MRC OX8 for rat CD8 antigen (Johnson et al. 1985); and MRC OX12 for rat B lymphocytes. Optimal conditions for each antibody were determined on tissues from normal animals. Control sections were processed with the rest but using phosphate-buffered saline instead of the primary antibody. The sections were fixed in acetone for 3 min, washed well with phosphate-buffered saline, incubated with or without the antibody overnight at 4°C, washed again and then incubated for 45 min with goat anti-mouse IgG (whole molecule) conjugated with alkaline phosphatase (Sigma, Poole, UK). Alkaline phosphatase was revealed by use of napthol-AS-MX phosphate, N-N dimethylamine and fast red (Mason and Sammons 1978). The presence of non-specific alkaline phosphatase reaction was checked by examination of sections prepared without primary antibody.

Rats were raised and handled in adherence with the Animals (Scientific Procedure) Act, 1986, UK Government, and procedures were carried out under appropriate Home Office project and personal licences.

Statistical significance was evaluated using one-way analysis of variance (ANOVA) and Student's *t*-tests when standard deviations were similar, but when differences existed Mann Witney U tests (MW) were employed instead. Means are given ± SEM. *P* values <0.01 are considered as significant.

Results

Serum tetraiodothyronine levels, body and thymus weights

The mean serum T4 concentration in control rats in the methimazole experiment was 57 ± 3 pmoles/l, with no significant difference between the values at 4 weeks and 6 weeks ($P=0.36$). However, serum T4 levels in control rats at 7 weeks (43 ± 3 pmoles/l) were lower ($P<0.02$; MW) than at 6 weeks (Fig. 1 a).

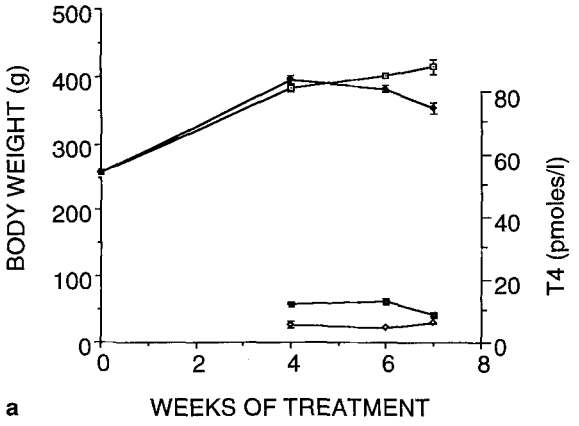
In methimazole-treated rats, mean serum T4 levels at 4 weeks (27 ± 5 pmoles/l) were significantly lower than in control rats sampled at the same time ($P<0.01$; MW). The difference between treated rats (63.3 pmoles/l) and control rats (23.1 pmoles/l) at 6 weeks was greater ($P<0.001$; MW) but by 7 weeks the difference was greatly reduced ($P < 0.04$; MW) because the control values were lower than at earlier times, and experimental values (30 ± 1 pmoles/l) were significantly greater than at 6 weeks ($P<0.002$).

In methimazole-treated rats the thyroid glands at 4 weeks were enlarged and mean follicular epithelial height (8 ± 1 μm) was less than in control rats (15 ± 1 μm, $P<0.001$; ANOVA, F ratio = 55.3). The follicles contained less colloid after 4 weeks of treatment, but at 7 weeks colloid content was increased and follicular epithelial cells were even more flattened (3–4 μm in height).

Mean body weight increased after 4 weeks in both carbimazole-treated ($P<0.0001$) and control ($P<0.0001$) rats although there was no difference between experimental and control animals either at the start or end of the 4 week period (time 0, 247 ± 1 g; 4-week-experimental rats, 288 ± 3 g; and 4-week-controls, 282 ± 2 g). At 4 weeks, the mean thymus weight in carbimazole-treated rats (226 ± 2 mg) was significantly lower ($P<0.0001$) than in control rats (472 ± 3 mg).

Similar changes were observed in methimazole-treated rats (Fig. 1 a). There were no significant differences in body weight between the groups of control and experimental rats at the start of the experiments (overall mean wt. 258 ± 2 g), or at 4 weeks (controls, 383 ± 6 g; methimazole-treated, 396 ± 6 g). At 6 weeks, control rats (401 ± 3 g) were significantly heavier ($P<0.01$) than methimazole-treated (382 ± 5 g). This trend increased at 7 weeks (controls, 415 ± 11 g; methimazole-treated, 354 ± 8 g; $P < 0.002$).

In the methimazole-injected rats, thymus weights at 4 weeks (387 ± 10 mg) were significantly reduced ($P<0.0001$) compared to controls, and continued to decline throughout the experimental period (6 weeks, 352 ± 16 mg, $P<0.0001$; 7 weeks, 302 ± 5 mg, $P<0.001$); thymus weights in control rats were similar (612 ± 4 mg) throughout the experiment (Fig. 1 b).



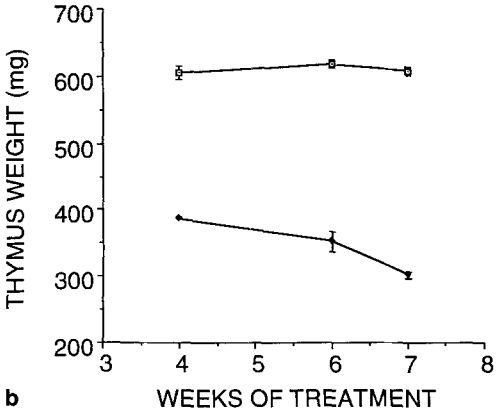


Fig. 1. a Body weights of control (□) and methimazole-injected (◆) rats with serum T_4 levels in control (■) and methimazole-injected (◇) rats. **b** Thymus weights from the same experiment. Control (□) and experimental (◆) rats. Mean ± SEM

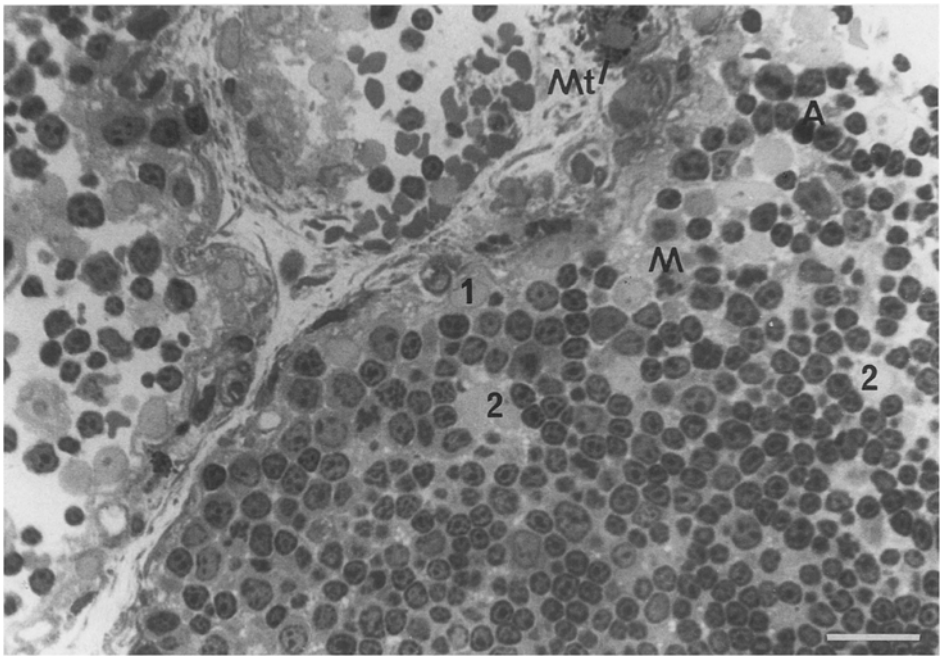


Fig. 2. Subcapsular and outer cortical region in a 1-μm-thick Epon-embedded thymus from a control rat. Methylene blue and azure II. *M* Mitotic figure; *1* type 1 epithelial cell; *2* type 2 epithelial cell; *A* apoptotic cell; *Mt* mast cell. *Bar*: 20 μm

Thymic structure

The thymuses of control rats (Fig. 2) were similar throughout the study period in both experiments. The cortex contained many thymocytes and some of these were in mitosis (91 ± 4 mitotic figures/mm^2). A few macrophages and apoptotic thymocytes were present. The medulla also contained numerous thymocytes, as well as interdigitating cells and macrophages. Mast cells were observed in the capsule.

The 6 epithelial cell types previously described by Wijngaert et al. (1984) were identified ultrastructurally. Type 1 cells lie around the gland and surround perivascular spaces of the cortex and part of the cortico-medullary junction (CMJ); types 2 and 3 cells are in the cortex; type 4 cells are in the deep cortex, the CMJ and occasionally in the medulla; type 5 cells are in the medulla; and type 6 cells are associated with small but distinct Hassall's corpuscles. Tonofilaments were particularly conspicuous in epithelial cell types 5 and 6, and small desmosomes were generally apparent between adjacent epithelial cells.

The majority of blood vessels in the thymic cortex were small blood capillaries, lined with continuous light and dark endothelial cells (Fig. 3). These capillaries in transverse section were surrounded by a thin ring of reticular fibres delimiting perivascular spaces 6–20 μm in diameter which contained mononuclear cells. Larger vessels were observed in the cortico-medullary zone and medulla with the largest in the septa.

In experimental animals, 6 weeks after the start of methimazole treatment the cortical capillaries had a greatly increased density of reticular fibres and the diameter of the perivascular spaces increased to approximately 20–55 μm. More reticular fibres were also found in the perivascular spaces around the other blood vessels,

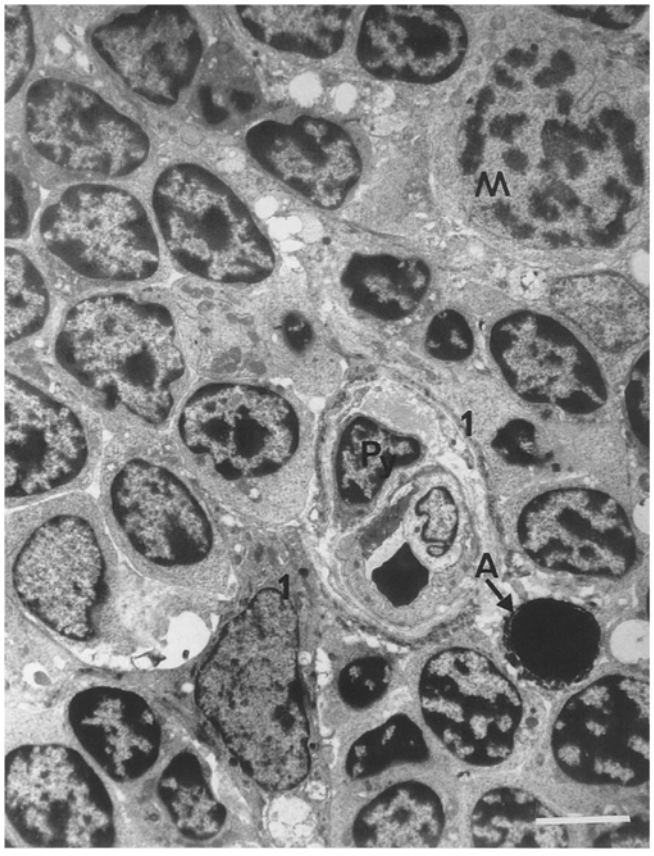


Fig. 3. Cortical capillary surrounded by a type 1 epithelial cell (*1*) from a control rat. The perivascular space contains a pericyte (*Py*). *M* Mitotic figure; *A* apoptic cell. *Bar*: 2 μm

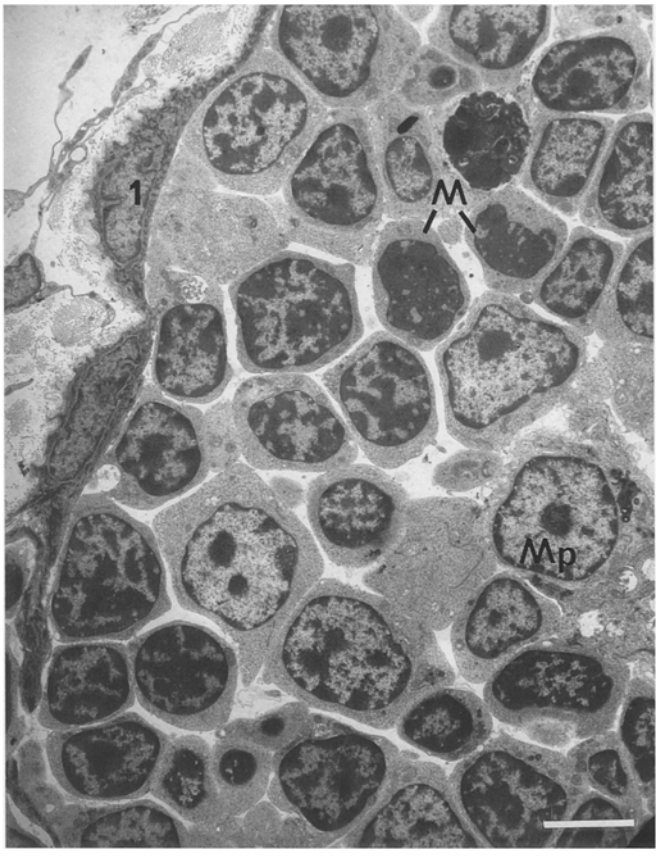


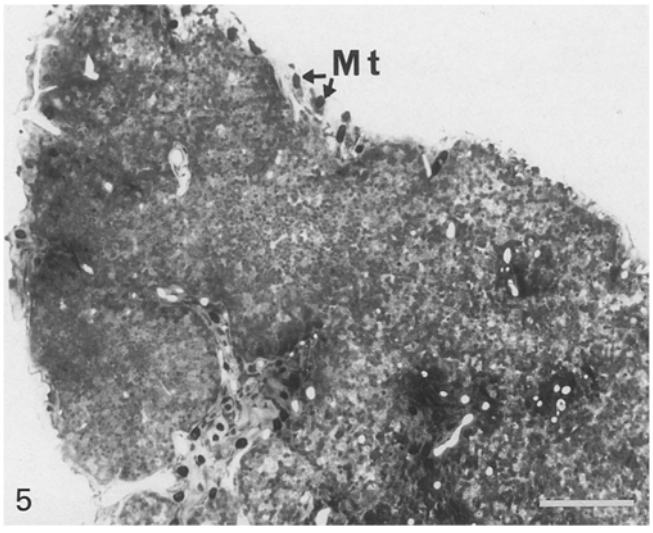
Fig. 4. Subcapsular cortex after 4 weeks of methimazole treatment. The subcapsular type 1 cells (*1*) are more electron dense and wrinkled on their outer surface than in control rats. *M* 2 cells with condensed chromatin typical of recently divided cells; *Mp* macrophage. *Bar*: 2 μm

and in the capsule and interlobular septa. Mononuclear cells were in the perivascular spaces especially around vessels at the CMJ and in the medulla. A few vessels with high walled endothelia were observed in most animals.

Thymic involution (atrophy) was found in rats killed after 4 weeks of both antithyroid drug treatments and was more marked after longer periods of drug treatment. Atrophied glands showed marked thymocyte depopulation in the cortex (especially the outer cortex, Fig. 4), increased apoptotic figures and macrophages, and decreased mitotic figures in the cortex (4 weeks methimazole-treated, $23 \pm 2/mm^2$, $P<0.0001$ compared with controls). The number of mitotic figures remained reduced for the rest of the experiment ($26 \pm 2/mm^2$ at 6 weeks; $29 \pm 4/mm^2$ at 7 weeks).

The depopulation of the cortex caused a decrease in distance between adjacent epithelial cell nuclei. These nuclei were 47 ± 3 μm apart in controls compared with 24 ± 2 μm after methimazole treatment for 4 weeks ($P = 0.002$), 20 ± 1 μm after 6 weeks ($P < 0.0006$). The distance decreased significantly between 4 weeks and 7 weeks ($P < 0.03$). After 6 weeks of methimazole, total thymocyte numbers ($4 \pm 1 \times 10^7$ cells) were significantly ($P < 0.0005$) reduced compared with controls ($8 \pm 1 \times 10^7$ cells).

The outer margin of the atrophied thymus became wrinkled (Fig. 4), the capsule and septa were thickened, and mast cells were often observed in the capsule. The longer exposures to methimazole (6 and 7 weeks) caused distortion of the thymic lobular structure and loss of distinction between cortex and medulla (Fig. 5). More acid phosphatase-positive macrophages were apparent throughout the gland. Most macrophages (especially those of the cortex) contained large phagocytic inclusions which could be identified as thymocytes in various stages of degradation (Figs. 6, 7) as well as vacuoles containing granular, concentric lamellar structures or membranous material. Some macrophages contained phagocytosed, degenerating epithelial cells with clearly defined tonofilaments (Fig. 7). The interdigitating cell population appeared to be increased in the thymic medulla (not quantitated). After drug treatment all types of epithelial cells, especially type 1 cells (Fig. 4), increased in electron density, and cortical epithelial cells (types 2, 3 and 4) had numerous intracytoplasmic vacuoles and cysts containing both electron-dense granular and fibrillar material along the inner surfaces of their limiting membranes (Fig. 8). Medullary epithelial cells contained multiple intracytoplasmic vacuoles and vesicles with microvillous projections of their walls (Fig. 9). Hassall's corpuscles, mainly



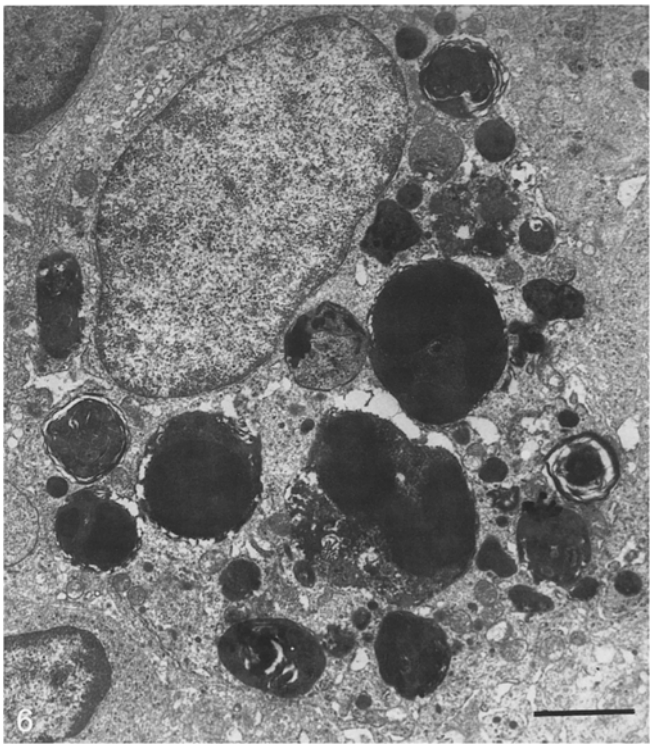


Fig. 5. Thymus after 7 weeks of methimazole treatment. 1 μm-thick Epon-embedded section stained with methylene blue and azure II. Note the loss of architecture and cortical depopulation. There are numerous mast cells (*Mt*) in the capsule and septa. *Bar*: 1 μm

Fig. 6. Cortical macrophage in a thymus after 6 weeks of methimazole treatment to illustrate the high degree of phagocytosis. *Bar*: 1 μm

formed by type 6 cells with numerous bundles of tonofilaments, were large in the hypothyroid animals.

After 6 weeks of methimazole administration, more numerous lymphocytes were seen around blood vessels and within the vessel walls of the deep cortex and CMJ (Figs. 10, 11). The high walled appearance of the endothelia was created by numerous lymphocytes within the blood vessels distorting the endothelial cells. At such sites the basal lamina between type 1 epithelial cells was discontinuous, and the layers of collagenous fibres disrupted. Most intramural lymphocytes had a cytoplasmic tail, but this was not oriented either predominantly adluminally or abluminally.

Increased exposure to methimazole caused a decrease in the numbers and intensity of staining of W3/13-immunoreactive (-ir) cells in both the cortex and the medulla, but there was an increase in both MRC OX35-ir and MRC OX8-ir cells around blood vessels, and MRC OX12-ir cells appeared within the blood vessel walls and lumina. In control rats there were few MRC OX12-ir cells and these were invariably in the medulla. Electron microscopy revealed occasional plasma cells in control rats, but many were observed in hypothyroid rats within the thymus, either singly or in groups, usually adjacent to blood vessels at the CMJ.

Discussion

The antithyroid compounds, carbimazole and methimazole, induced hypothyroidism and caused thymic atrophy. The occurrence of thymic atrophy or involution after stress (e.g. severe illness, malnutrition and after treatment with drugs or hormones) is well established and it has even been suggested that atrophy may be beneficial to the immune status (Aronson 1989). Stress involution is thought to be mediated by a direct effect of adrenal glucocorticoids on thymocytes causing death of mainly small cortical thymocytes by apoptosis (see Kendall 1990). This can occur within 3–4 h of glucocorticoid injection (Cowan and Sorenson 1964; Wyllie 1980) and remnants of the apoptotic cells remain within macrophages for up to 24 h (Leene et al. 1988). Whilst the thymic atrophy reported here is morphologically similar to stress-induced atrophy (Dougherty and White 1945; Weaver 1955; Ito and Hoshino 1962; Cowan and Sorenson 1964) it is unlikely to be caused by the stress of injection alone as the thymus of control animals was not atrophied.

Carbimazole is a carbethoxy derivative of methimazole which is converted into methimazole in the body. Methimazole is a highly potent antithyroid drug with a long duration of action. Whilst their actions on the thyroid have been documented (Goth 1981; Gilman and Murad 1980), there is no data on the effect of carbimazole or methimazole on the thymus. Höhn (1959) considered that the reduction of thymic weight in domestic hens after thiouracil treatment resulted from the hypothyroid status rather than from a direct action of that chemical.

Methimazole injections to rats caused a reduction in serum T4 within 4 weeks due to the blocking effect of these antithyroid drugs (Berthier and Lemarchand-Beraud 1978; Männistö et al. 1979; Redmond and Tuffery 1981). Propylthiouracil administration causes depression of thyroid hormone levels accompanied by growth hormone deficiency (Lewis et al. 1965) and growth hormone and thyroxine have synergistic effects on lymphoid tissues (Baroni et al 1969). Thus the thymic effects of antithyroid drugs might be due to disturbances of hormones other than the lack of thyroid hormone.

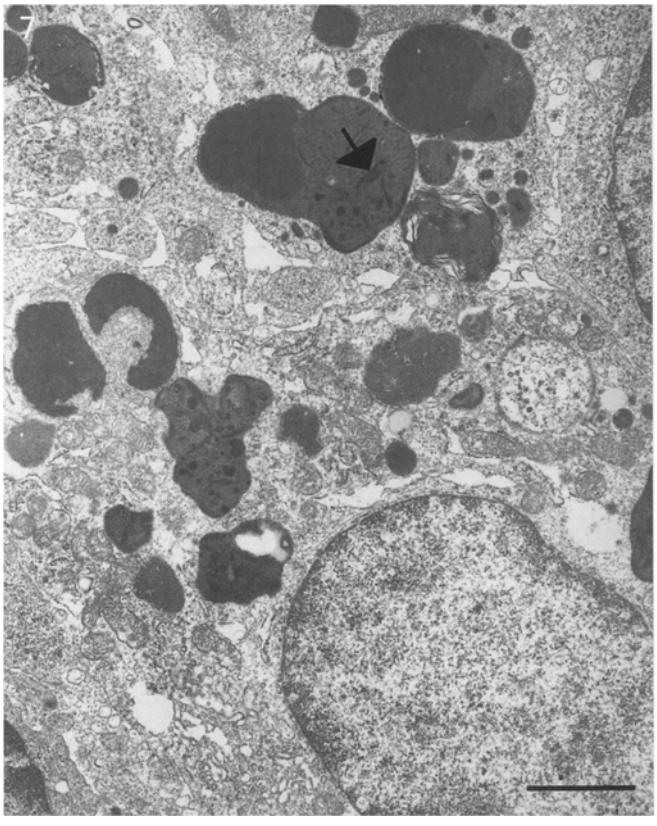


Fig. 7. Cortical macrophages in a thymus after 6 weeks of methimazole treatment. Some of the heterogenous inclusions contain bundles of tonofilaments (*arrow*). *Bar*: 1 μm

Fig. 8. Epithelial cell with numerous vacuoles (*V*) in its cytoplasm from the deep cortex of a thymus after 7 weeks of methimazole treatment; dense bundles of tonofilaments (*arrow*). *Bar*: 1 μm

The reduction in thymus weight induced by carbimazole and methimazole was mainly due to a reduction in thymocyte numbers. The reduced number of mitotic figures suggests that this may result from reduced proliferation, but mitosis could still be detected even in the progressive stage of thymic involution. Phagocytosis was increased but did not appear sufficient to account for the loss of thymocytes. Therefore, methimazole may also have increased the migration and release of thymocytes into the medulla and/or peripheral circulation as suggested for hydrocortisone-induced thymic involution (Ito and Hoshino 1992).

Prolonged administration of carbimazole or methimazole depopulated the cortex of thymocytes but the medulla was less affected. Such an inversed appearance of the thymus occurs in cyclophosphamide-induced thymic involution (Milicevic et al. 1984), and in old individuals (Steinmann 1986). The drugs could possibly have a selective effect on the cortex (there is no data on this point); alternatively, the persistence of medullary thymocytes could be due to continued recruitment of cells from the cortex despite the cortical atrophy, to a lack of release of medullary cells, or to re-entry of circulating cells into the medulla. The high level of cortical apoptosis in drug-treated animals suggests that failure to release medullary cells and/or re-entry is the most probable explanation. The reduced numbers of T (W3/13-ir) cells in both cortex and medulla makes a retention of medullary cells unlikely. The entry of peripheral B (MRC-OX 12-ir) cells into the thymus was frequently observed, but the labelled T and B cells did not account for all medullary cells. Further studies with other antibodies are required to characterise the medullary thymocytes in this experimental situation.

The three main areas of the thymus (cortex, CMJ, medulla) are each characterised by their own population of macrophages (Duijvestijn and Hoefsmit 1981; Brelinska et al. 1985; Milićević et al. 1987) and this was observed in both the control and drug-treated animals. The apparent increase in cortical acid phosphatase-positive macrophages after drug treatment is similar to that found by Kahri et al. (1965) in other forms of thymic involution, except that the engulfing of epithelial cells is a novel observation for thymic macrophages. This could imply an extensive restructuring of the thymus in chronic hypothyroidism.

Alterations to the ultrastructural appearance of epithelial cells after drug treatment included features that may indicate cellular ageing (increased density, etc.) to evidence of phagocytosis (generally of thymocytes) in cortical cells (Haelst 1967; Dourov 1974; Clarke and Kendall 1989) which is not generally observed in normal healthy thymuses.

In methimazole-induced involution, the Hassall's corpuscles became hypertrophied and more frequently contained central necrotic debris, as reported for X-irradiat-

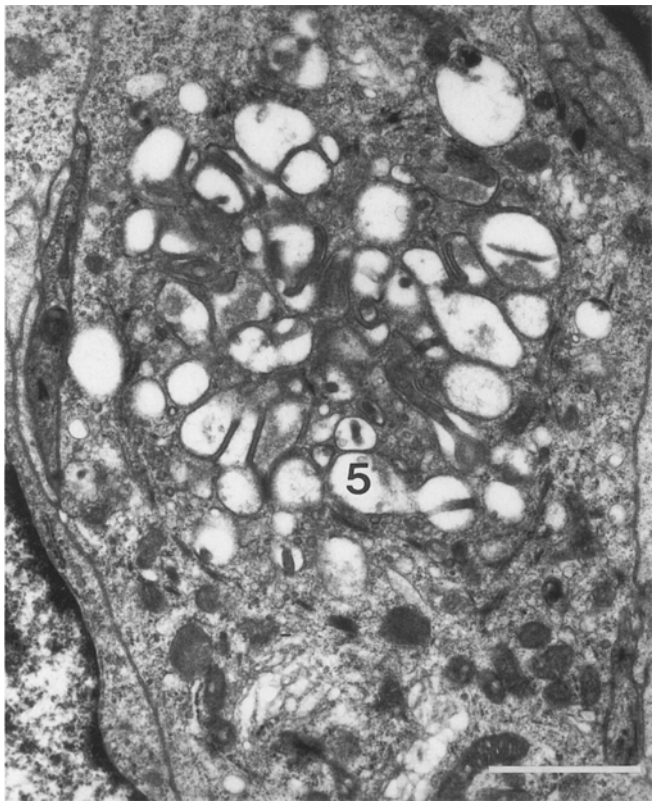


Fig. 9. Numerous vacuoles in a medullary type 5 epithelial cell (5) after 7 weeks of methimazole treatment. *Bar*: 1 μm

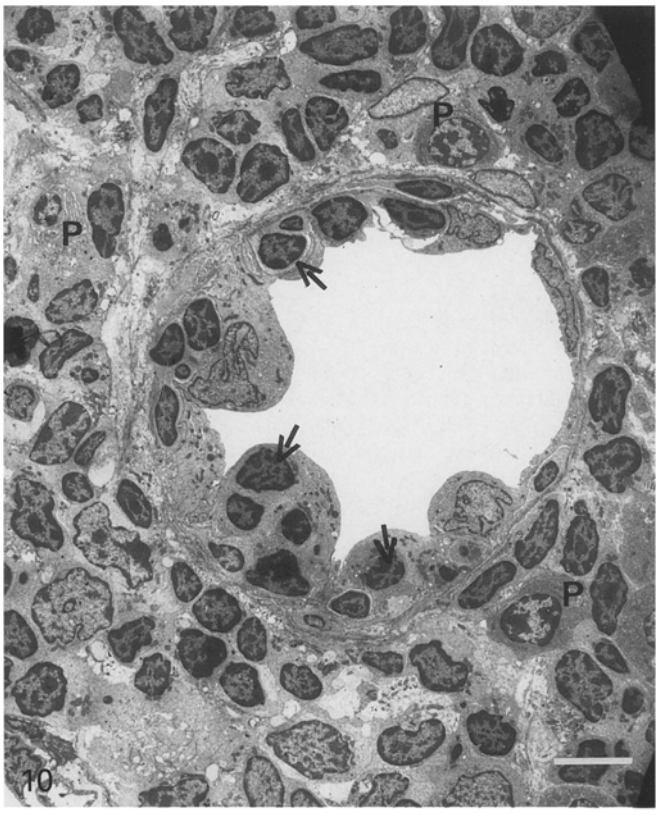


Fig. 10. Blood vessel at the corticomedullary junction 6 weeks after methimazole treatment. Intramural lymphoid cells (*arrows*). *P* Mature plasma cells close by in the gland. *Bar*: 1 μm

Fig. 11. Intramural lymphocyte in the corticomedullary region of a thymus after 6 weeks of methimazole treatment. The abluminal cytoplasmic tail containing organelles indicating movement from the thymus to the vessel lumen. *Bar*: 1 μm

ed thymus glands (Blau 1967), starvation, treatment with steroids (Siegler 1964), or adrenocorticotropic hormones (Dougherty and White 1945). Conversely, Baker et al (1951) reported that Hassall's corpuscles were rarely found in involuted rat thymuses.

An intriguing finding of this study was the apparent movement of B cells through venules at the CMJ and the accumulation of small groups of mature plasma cells nearby. High walled endothelial post-capillary venules (HEV) are considered to be important sites for the migration of lymphocytes in lymph nodes (Gowans and Knight 1964), and also in the thymus (Pabst et al. 1989) although only a few HEV are present. The morphology of the vessel walls showing lymphocyte migration is very similar to that reported by Törö and Olah (1967); they were not lined with high walled endothelial cells. The lymphocytes appear to migrate in both directions. The increased number of lymphocytes migrating through blood vessel walls during methimazole-induced thymic involution raised the possibility that thymic vascular permeability is increased as suggested in autoimmune mice (Kotani et al. 1988), in induced-involution where the fate of iodinated serum proteins was examined (Blau and Veall 1967), and in ageing (Dumont et al. 1982).

The presence of plasma cells within the thymic parenchyma, of B lymphocytes around thymic blood vessels, and the movement of lymphocytes through thymic blood vessels suggest that the plasma cells may have originated outside the gland. B cells are present in, or enter the

thymus in other conditions (Ghossein et al. 1963; Sainte-Marie 1965; Goldstein 1966; Moorhead et al. 1974; Spencer et al. 1992), but the significance of this phenomenon remains to be explored.

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