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The noradrenergic innervation of the rat thymus during pregnancy and in the post partum period

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ABSTRACT

The noradrenergic innervation of the rat thymus during pregnancy and the post partum period was examined by a sucrose glyoxylic acid method for catecholamines, and by high pressure liquid chromatography. Fluorescent nerves decreased in number throughout pregnancy when there was an overall loss in thymic weight due to cortical involution. These changes are maximal by parturition. There was a dramatic increase in nerves between d 21 of pregnancy and d 1 after parturition, especially in the capsule and around blood vessels in the connective tissue septa. The neonates were removed at parturition and thymic weight was rapidly regained. The increased numbers of nerves remained throughout this post partum period.

Noradrenaline levels in the thymus altered in a similar pattern throughout pregnancy and the post partum period, but did not parallel thymic weight changes. The mean noradrenaline concentration in the virgin thymus was 1063 ± 107 pg/mg protein. Levels remained similar during early pregnancy and increased significantly at d 16. Virgin levels were regained by d 21. Values peaked after parturition but rapidly decreased over the next 3 days, and remained at or below virgin levels to d 28 except for a transient rise at d 10 post partum. Adrenaline values were consistently below detection levels.

This study shows that there are variations in both nerves visualised, and in neurotransmitter (noradrenaline) content in the thymus during the course of pregnancy and the post partum period. Thus thymic function could be influenced by central events (levels of catecholamines in peripheral blood) as well as local events mediated by transmitter changes in nerves.

Key words: Catecholamines; noradrenergic nerves.

INTRODUCTION

The early descriptions of thymic innervation, based on silver staining methods (Brauecker, 1923; Hammar, 1935; Knocke, 1955) showed that the nerves in the embryo grew from the phrenic and vagus nerves into the developing thymus. This and later observations (Bulloch, 1985, 1988) have implicated the nerves in the process of growth and development of the organ. Later studies have shown that the midcortex, where most thymocytes reside, is relatively free of nerve profiles (Bulloch & Moore, 1981; Al-Shawaf et al. 1991) whereas the subcapsular cortex and cortico-medullary junction contain discrete nerve nets (Kendall & Al-Shawaf, 1991). These are primarily sites of cellular egress and development of early thymocyte precursors (reviewed in Kendall, 1991). β-adrenergic receptors have been identified on thymocytes, and catecholamine stimulation induces differentiation markers on prothymocytes (Singh & Owen, 1976; Singh, 1979; Singh et al. 1979). In many organs, adrenoreceptors on blood vessels control the degree of vasodilation. Neurotransmitter release in the thymus therefore may have functional influences on its architecture, the immigration of prothymocytes, thymocyte differentiation and thymocyte release.

During pregnancy and the post partum period, the thymus undergoes dramatic changes in most mammals

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(reviewed in Kendall & Clarke, 1994). Not only does it lose two-thirds of developing thymocytes, but the medulla becomes more active. The significance of these changes for pregnancy remains to be clarified, but such radical rearrangements are likely to have effects on the maternal immune system and to influence her ability to protect the fetus from harmful immune responses to paternally transmitted antigens. This study was therefore designed to examine the effects of these natural physiological states on the innervation of the organ and its neurotransmitters. Such a study reduces the amount of artificial interference that is often associated with animal models. On the other hand, pregnancy is a complex event so that the cause and effect of many changes observed here will now have to be further explored.

MATERIALS AND METHODS

Female Porton Wistar rats, 3–4 months of age, were mated and checked daily for the presence of vaginal plugs. The date of observation of a plug was designated as d 1 of pregnancy. Groups of 5 were killed on each of d 4, 14, 16, 18 and 21 of pregnancy (16–24 wk of age at autopsy). Five virgin females (15–17 wk of age) and 5 males (16–17 wk old) were used as controls. In a second experiment, the rats were allowed to give birth. They were checked twice daily for signs for parturition and the neonates were removed at 09.00 h on the first morning after delivery, to reduce the confounding factor of different lactation times. This was designated as d 0 post partum. Groups of 5 were killed on each of d 1, 2, 3, 7, 10 (n = 3), 14, 21 and 28 post partum (15–18 wk of age at autopsy). Another group of 5 virgin females (13 wk of age) provided controls for this experiment.

Animals were killed by asphyxiation with halothane or carbon dioxide and the thymus was dissected out and weighed; the 2 lobes were separated and each was cut in half. The inferior portion of the left lobe was frozen in liquid nitrogen for high pressure liquid chromatography (HPLC) and protein analysis. The inferior portion of the right lobe was frozen in isopentane, precooled in liquid nitrogen, and used for microscopic evaluation. All samples were stored at −70 °C until further processing.

Microscopy

Tissues from 3 animals from selected groups were examined by fluorescence microscopy to study the distribution of nerves within the thymus. From each block, 6 serial sections (20 μm) were cut on a cryostat and mounted on uncoated slides. The first and last were stained with haematoxylin and eosin (H & E) for general histology and the other 4 were stained for catecholamines with a sucrose–phosphate–glyoxylic acid (SPG) method modified from de la Torre (1979). Each slide was thawed for 30 s at room temperature, and dipped 3 times (1 s per dip) in the SPG solution (pH 7.4). Excess solution was wiped off and the slides were dried under a cool air stream from a hair dryer for 20–30 min. Each dried section was covered with a drop of paraffin oil, heated in an oven for 2.5 min at 90 °C and coverslipped. Stained slides were wrapped in foil and stored at 4 °C until analysed. The sections were examined with a ×40 objective on a Nikon fluorescence microscope equipped with the filter combination EX405/410, DM430 and BA435.

For each block sectioned, a tracing was made of an H & E-stained section which was enlarged with a photographic projector (×35). This was used as a template on which to make a composite drawing of all nerves observed on the 4 serial sections stained with SPG.

HPLC and protein analysis

A frozen half-lobe from each animal at all time points was homogenised in 500 μl 0.1 M perchloric acid, chilled at 4 °C for 1 h and spun at 13000 rev/min for 5 min. The supernatant was removed and stored at −20 °C for catecholamine estimation. As the weight of tissue used for HPLC was not consistent, all samples were assayed using a modified Lowry method (Markwell et al. 1978) to determine the total protein content. For this procedure, the pellet was resuspended in a further 500 μl 0.1 M perchloric acid and sonicated immediately prior to being assayed.

Chromatography was performed using a Waters 501 pump, a Gilson 231/401 autosampler injector and a Waters 460 electrochemical detector. Integration was carried out by a Hewlett Packard HP3396A integrator. The column was spherisorb 15 cm × 4.6 mm with 3 μm packing (Phase Separations Ltd, Clwyd, UK). The buffer used was chloroacetic acid (0.085 M) with 150 mg/l octane sulphonic acid, 100 mg/l EDTA and 3% methanol. The pH was adjusted to 3.0. Flow rate was 1 ml/min and the column was thermostated to 35 °C. The detector had an oxidation potential of +0.77 V. The injection volume was 20 μl and the results of the test samples were correlated to a standard curve of 200–800 pg noradrenaline (NA) (Sigma, A7257), 100–400 pg adrenaline (Sigma, E7386) and 50–200 pg dopamine (Sigma, H8502). All standard curve values are

expressed as amount per 20 µl injected into the column. The amount of NA detected by HPLC was then expressed as pg NA/mg protein so that all samples were comparable.

Statistics

Means are given ± standard errors (S.E.). Data were analysed by 1-way analysis of variance (ANOVA). Comparisons between individual data groups were analysed by Student's t test. Differences are considered as marginally significant ($P < 0.1$), significant ($P < 0.01$) or very significant ($P < 0.001$).

RESULTS

There was no significant difference between the body weights of the 2 groups of virgin females (236 ± 6 g and 214 ± 8 g) but their combined mean weight (225.6 ± 6 g) was very significantly different ($P < 0.0001$) from that of the males (420 ± 12 g) of a similar age. Mean body weights during pregnancy and the post partum period are shown in Figure 1. Parturition generally occurred on d 23 so this was used on all graphs to separate the pregnant and post partum results.

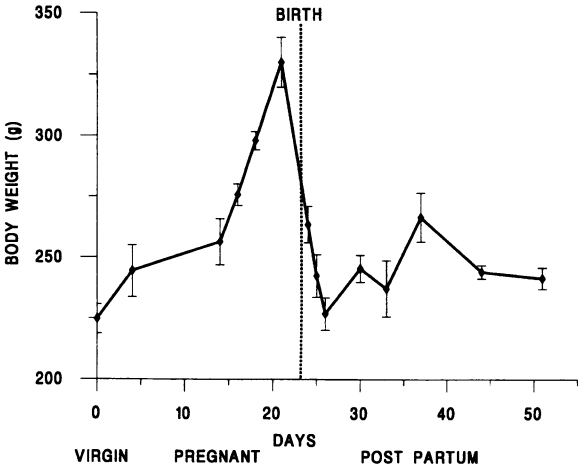

Fig. 1. Mean (± S.E.) body weights of rats sampled during pregnancy and the post partum period. Values during pregnancy include the weight of the embryos and associated fetal membranes and fluids.

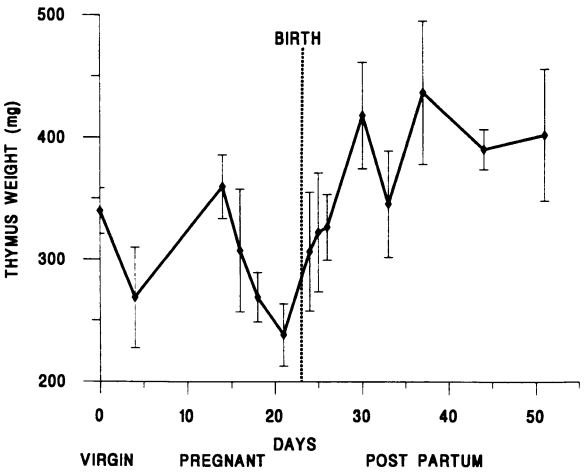

Fig. 2. Mean (± S.E.) thymus weights of rats sampled during pregnancy and the post partum period.

Except at d 4 of pregnancy, viable embryos were counted at the time of autopsy and neonates on the day of birth. The mean number of embryos per pregnant rat was 13 ± 0.3 and litter size at birth was 12 ± 0.3.

The mean thymus weight for each group of virgins was not significantly different (350 ± 34 mg, and 328 ± 19 mg) but their mean combined weight (340 ± 19 mg) was less ($P = 0.04$) than that of the males (438 ± 49 mg). Although there was considerable variation in thymic weight at each sampling point (Fig. 2), ANOVA analysis showed a marginally significant variation throughout the study period ($F = 2.102$, $P = 0.02$, D.F. = 72). There was an overall significant decrease during pregnancy (virgins to d 21, $P = 0.007$) with a consistent decline in late pregnancy between d 14 (359.4 ± 26.1 mg) and d 21 (238.2 ± 25.28 mg; $P = 0.01$). From d 21 of pregnancy to d 7 post partum (417.9 ± 43.62 mg), thymus weight increased significantly ($P = 0.007$) and thereafter remained constant at a level slightly greater than in virgins.

Microscopical analysis

The relationships of the nerves to the various components of the thymus, made by tracing a projected image of an H & E-stained section from each block, are shown in Figure 3. It can be seen that a thymic lobe, in cross-section, is divided into smaller lobules by connective tissue septa which contain blood vessels. Other septa extend from the outer capsule or from the interlobular septa to the corticomedullary junction (CMJ). Each lobule consisted of an inner medulla separated by the CMJ from an outer cortex and the entire lobule was enclosed in a capsule. On the SPG-stained sections, the identification of the cortex was simplified by the presence of yellow cortical autofluorescent cells (CAF, as defined by Ackerman et al. 1991). A concentration of these cells at the CMJ allowed this region and the almost CAF-free medulla to be easily recognised. The nerves could be distinguished by their specific blue fluorescence. The location and density of the nerves at different stages, based on the composite drawings of each set of 4 serial sections, is shown in the Table.

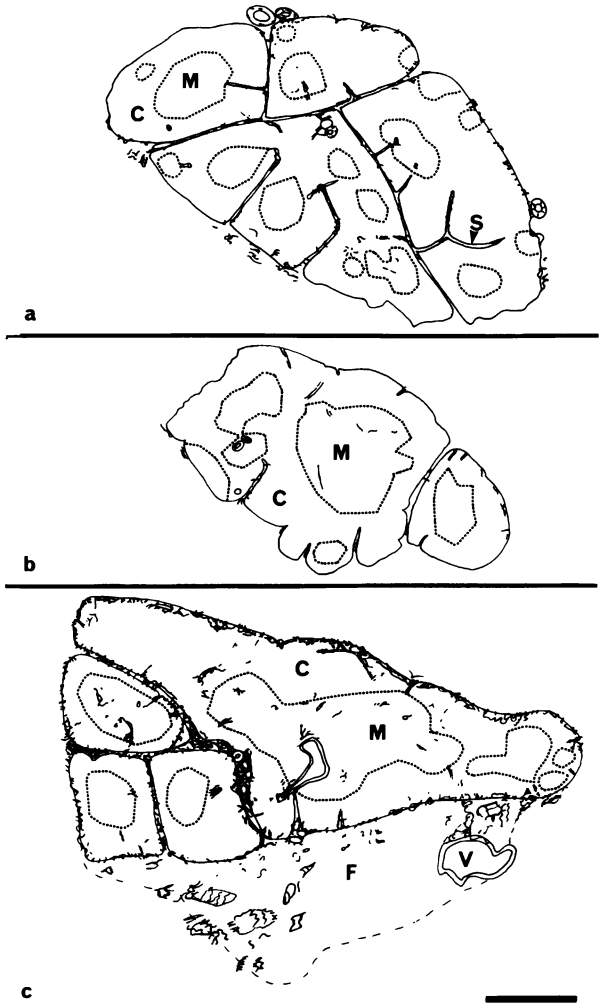

Fig. 3. Composite drawings of the nerves observed on 4 serial SPG-stained sections. (*a*) Virgin control; (*b*) d 21 pregnant; (*c*) d 1 post partum (d 24). C, cortex; F, fat; M, medulla; S, septum; V, blood vessel; ----, ring of yellow autofluorescent cells marking the CMJ; nerves are shown as short discontinuous lines, with larger irregular profiles associated with blood vessels in the fat. Bar, 1 mm.

Virgin

In virgin controls, the innervation of the capsule was unevenly distributed, with large areas having no nerves visualised by this technique. When present, the nerves were short, fine and wavy, and some crossed the capsule into the subcapsular cortex, whilst others undulated in the plane of the capsule parallel to the outer surface (Fig. 4*a*). Occasional fine nerves were observed in the cortex but, at the resolution available, it was not possible to determine if they were truly cortical, or were actually in the connective tissue around small blood vessels. The septa were well innervated and 2 types of nerves were observed. Some occurred as branching bundles of fibres (Fig. 5*a*) and others formed plexuses around the blood vessels which passed along the septa (Fig. 6*a*). Some blood vessels also crossed the CMJ into the medulla, and nerves were occasionally observed in conjunction with these vessels (Fig. 7*a*). In cross-section the medulla appeared as an irregularly shaped patch surrounded by cortex. Nerves were observed in less than half of these areas of medulla and a similar number were associated with the CMJ.

Pregnancy

Throughout pregnancy, no change was observed in the nerves of the cortex, compared with the virgins. The numbers of nerves in both the medulla and the CMJ decreased slightly in the 2nd half of pregnancy (medulla by d 14 and CMJ by d 16). The density of septal nerves was lower on d 21. In the capsule, there was a slight increase during midpregnancy to a maximum at d 18, and then a decrease to virgin levels by d 21.

Post partum period

The size and density of nerves changed most dramatically between d 21 of pregnancy and d 1 of the post partum period. The only compartment unaffected was the cortex, which consistently contained a very few, fine nerves which may or may not have been associated with small blood vessels. Throughout the post partum period examined, there were consistently more nerves crossing or associated with the CMJ (Fig. 7*b*) than during pregnancy or in the virgins. The nerves in the medulla were similarly increased, both in number and in size, throughout the post partum period, so that it was rare to observe any medullary region without nerves. The septa, which contained only a few nerves at the end of pregnancy, were completely different on d 1 of the post partum period. All septa contained so many nerves that the entire region was fluorescent. Detailed examination revealed extensive nerve plexuses around the blood vessels (Fig. 6*b*). This level of innervation in the septa decreased slightly by d 7 and 10, then increased again to remain at peak density (Fig. 5*b*) until the end of the study at d 28 post partum. Even greater changes occurred in the capsule, which had only contained a few nerves in virgins and on d 21 of pregnancy. On d 1 of the post partum period, the capsule (fig. 4*b*), like the septa, contained very many fluorescent fibres. In this region the nerves were independent of blood vessels with occasional branches as seen in virgins, although the density of nerves was greatly increased. By d 3, slightly fewer nerves were seen and this trend continued so that the innervation on d 7 resembled

Table. *Distribution of SPG-positive nerve profiles in the different regions of the thymus during pregnancy and the post partum period (day of parturition = d 23)*

Day	Capsule	Cortex	Septa	CMJ	Medulla
Virgin	1–2	1	3	1–2	1–2
4	1	1	2–3	1–2	1–2
14	2	1–2	3	1–2	0–1
16	2	1–2	2–3	0–1	1–2
18	2–3	1–2	2–3	1	0–1
21	1–2	1	2	1	1
24	5	1–2	5	2–3	3
26	4	1–2	5	3	3
30	1–2	1–2	3	3	3
33	2–3	1	3–5	2–3	3
37	3	2	4	3	3
51	2–3	1	4–5	3	3

1, Only occasional nerves; 2, a few nerves consistently observed; 3, moderate number of nerves observed; 4, many nerves; 5, so many nerves that the fluorescence almost covers the entire region.

that of the virgins. From d 10 to 28 the density of nerves in the capsule was slightly higher than in the virgins.

HPLC results

In all the samples adrenaline was below detection level, dopamine was either undetectable or present at extremely low levels and NA was always present.

There was no significant difference in the NA content (nor body or thymus weights) of the thymus in the 2 groups of virgins. Therefore, in all graphs and analyses of results, the data from the 2 groups of virgin females were combined. The mean NA concentration in the thymus of virgin female rats was 1063 ± 107 pg/mg protein. ANOVA analysis showed significant changes ($F = 2.849$, $P = 0.003$, D.F. = 72) in NA content throughout pregnancy and the post partum period (Fig. 8). The changes did not parallel those of thymus weight. After a slight decrease during early pregnancy (virgins to d 14, $P = 0.09$), there was a significant increase at d 16 (d 14–16, $P = 0.009$), followed by an immediate decrease again to virgin levels at d 21 (d 16–21, $P = 0.01$). A nonsignificant increase in NA occurs 1 day after parturition (d 21 of pregnancy to 1 post partum, $P = 0.15$), followed by a rapid decrease for the first 3 days post partum (d 1–3, $P = 0.04$). Thereafter, except for a transient increase at d 10 (d 3–10, $P = 0.03$) and decrease at d 14 post partum (d 10–14, $P = 0.01$; virgin–d 14, $P = 0.08$), the concentration of NA returned to virgin levels for the duration of the period studied. The d 16 pregnancy NA levels may not be as significantly raised as shown in Figure 8 since the values are greatly enhanced by those of a single female.

DISCUSSION

The loss in thymic weight in pregnant female rats after midpregnancy is in accordance with other studies (Leeming et al. 1984, 1985; Habbal & McLean, 1992). In mice it has been shown that the number of embryos may be one factor to influence this process (Clarke, 1984), but the variation in embryo number was so small in this study that this factor is unlikely to have influenced the results. Thymus weights were significantly increased by d 7 post partum, and remained similar thereafter. This was anticipated since it is known that thymic weight is normally regained after lactation ceases (Grégoire, 1947).

There were changes both in the nerves seen throughout this period and in NA analysed by HPLC. Before discussing the implications of these findings however, it is necessary to consider the significance of the results themselves.

There are a number of factors that may affect the levels of NA. Strain differences, the handling of animals and the method of sampling could all contribute to variations. Popper et al. (1977) compared the levels of NA and adrenaline in rats after a relatively unstressful technique for obtaining blood (cannulation) compared with decapitation, and during various physiological states including postoperative periods. Handling or restraining prior to taking blood both greatly increased plasma catecholamine levels. In particular, lifting rats by the tail (a recommended method of handling rodents) increased plasma adrenaline levels. Decapitation without anaesthesia caused about a 3-fold rise in plasma NA concentrations within 10 s of collecting blood, and levels continued to rise thereafter.

An earlier study of thymic NA content (Bellinger et al. 1988, 1989) recorded stable NA content from 8 to 27 months of age in Fischer 344 rats although metabolites of NA did change. Values were graphed in pmol per thymus, per g wet weight and per mg protein. These values convert to levels approximately 3 times higher than those in this study. Bellinger et al. (1988) decapitated the rats before excising the thymus, so the differences are explicable. On the other hand del Rey et al. (1981) anaesthetised the rats and then estimated thymic catecholamine concentrations using a radiometric–enzymatic assay and recorded 82.89 ± 8.19 ng NA/g tissue in germ-free female Holtzman rats weighing 250–300 g. The virgin female rats we sampled were a little lighter in weight but our

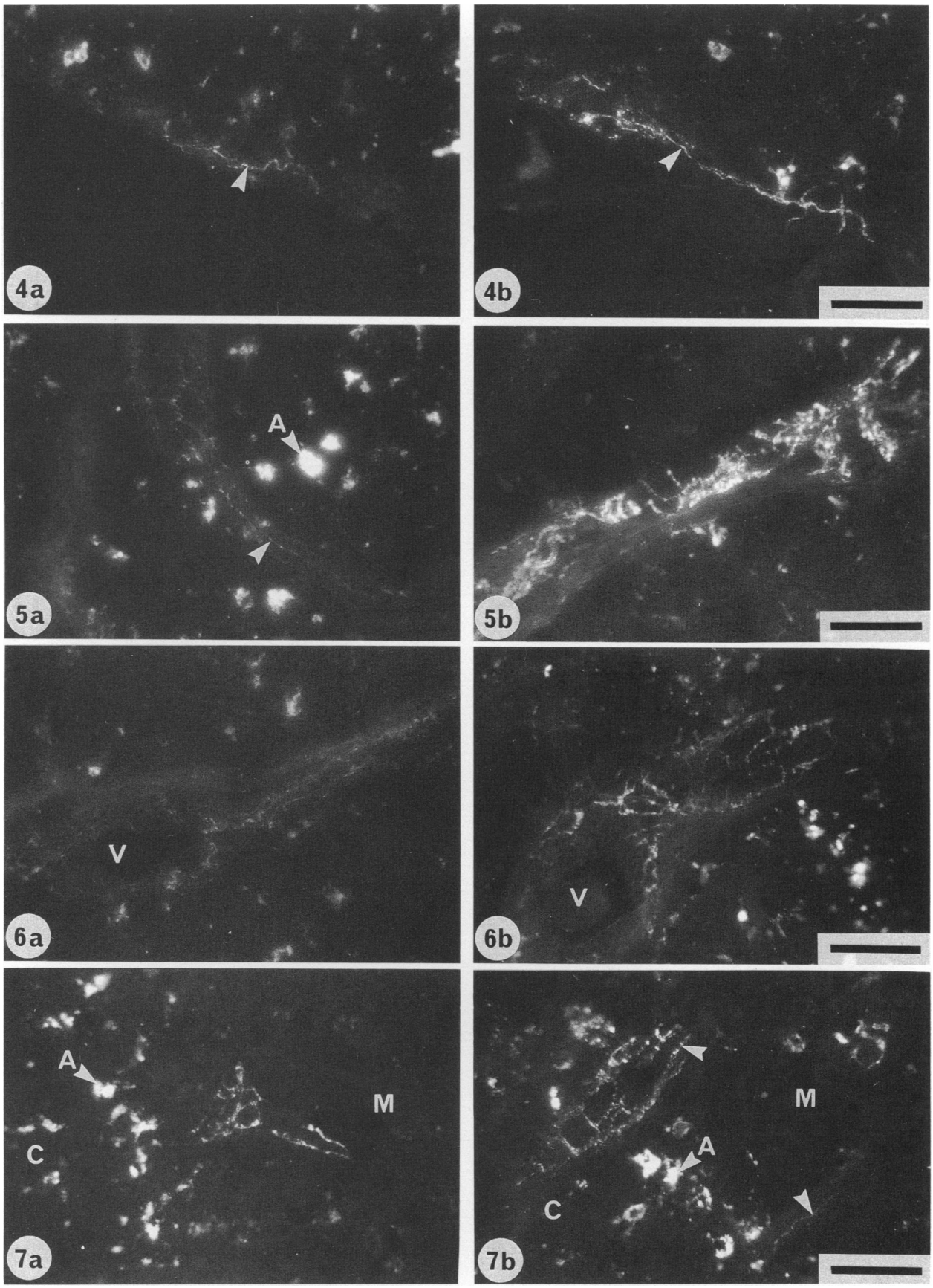

Fig. 4. Nerves in the thymic capsule. (*a*) Virgin control; (*b*) d 1 post partum. Arrowhead, nerve. Bar, 50 μm.

NA concentrations are only slightly higher, assuming that the protein content is approximately 10% of wet weight.

In this study, since care was taken to anaesthetise the rats gently, and HPLC adrenaline levels were undetectable, we conclude that this measure of the stress response was minimal.

The SPG method for noradrenergic nerves in tissues has been in use for a number of years. The localisation of NA and dopamine within nerve terminals and varicosities (Lindvall et al. 1981) has a 10-times greater sensitivity (Lindvall & Björklund, 1974) than the older formaldehyde reaction (FA) based on Falck et al. (1962). It has been estimated that the FA method can visualise these catecholamines at 1–5 pg/μg protein (Jonsson, 1971), so values of 5×10^{-7} pmol of dopamine in varicosities have been suggested as being readily detectable (Lindvall et al. 1981).

In spite of the difficulty in quantitating nerve profiles, it is clear that the thymus has many more nerves visible both in the septal and subcapsular/capsular regions immediately after parturition, and throughout the post partum period than in virgins and during pregnancy. These results do not accord with the HPLC NA results. One explanation could be that, although NA may be present in thymic nerves, NA can also reach the thymus through the circulation, which would influence the concentrations found by HPLC.

Dramatic changes in metabolism occur at parturition. In humans, plasma NA (and adrenaline) rise during labour and after delivery (Lederman et al. 1977). Thus the rise in NA in the thymus, if it is in the vascular compartment, may be a reflection of this. The hormonal changes that accompany the post partum oestrus (within 24 h of parturition) and the later reestablishment of regular oestrous cycles could also affect NA blood levels. Cyclic variations have been reported in the innervation of the rat reproductive organs (Adham & Schenk, 1969) although more recent studies have failed to confirm this (Melo & Machado, 1993).

The innervation of other organs has been studied by SPG, and the NA content determined by HPLC. In the uterus, nerve profiles are also decreased at the end of pregnancy in many animals (Rosengren & Sjöberg, 1968; Owman et al. 1975; Sporrung et al. 1981), but not in rat and human (Lederman et al. 1977). The pancreas and other nonreproductive system tissues do not show changes in innervation during pregnancy. In the guinea pig the adrenergic nerve profile decrease of the uterus has been shown to be due to degeneration of fibres (Sporrung et al. 1981). This is followed by a post partum recovery in the level of innervation (as seen in our studies), but it is much slower (Alm et al. 1982) than in the rats studied here. Thus we conclude that the changes in NA, as visualised by induced fluorescence, are due to alterations in transmitter content rather than degeneration or regrowth.

At the end of pregnancy the thymus is at its minimum weight. The increased presence of nerve profiles found after parturition, coincides with the regrowth of the thymus. Previous studies have suggested that the innervation of the thymus is necessary for its development and growth (reviewed in Bulloch, 1985), and this might also be the case here. It is interesting that there is also a rise in NA at d 16 of pregnancy when an enlargement in the medulla of the thymus has been found in mice (Clarke et al. 1994) and in rats (work in progress).

Catecholamines could also have other functions in the thymus (Livnat et al. 1985), particularly since noradrenergic nerves seem to be of two types: those associated with blood vessels and those independent of the vasculature. The presence of adrenergic receptors on blood vessels is well known, and Cowen (1984) found that the proximity of NA-containing nerves to smooth muscle appears to be related to functional neurotransmission. Thus where there is an intimate juxtaposition of nerves and blood vessels, changed levels of NA could influence lymphocyte trafficking since the β-action of catecholamines is known to decrease the frequency and force of smooth muscle contraction in lymphatics (Allen et al. 1986). Nerve stimulation (Webber et al. 1970) and the injection of NA (Foa, 1943) also cause the release of blood cells from bone marrow. However, only at mid pregnancy does an increase in NA level occur before a loss of thymic weight; at parturition the NA increase is followed by thymic weight increases, when cellular egress is not expected unless proliferation exceeds egress. At d 10 post partum when NA levels are increased, thymus weights have stabilized.

The nerves that do not form a plexus around the

Fig. 5. Typical septal nerves. (*a*) Virgin control; (*b*) d 14 post partum. A, yellow autofluorescent cell; arrowhead, nerve. Bar, 50 μm.

Fig. 6. Large blood vessels with their associated nerve nets. (*a*) Virgin control; (*b*) d 3 post partum. V, blood vessel. Bar, 50 μm.

Fig. 7. (*a*) Vascular-associated nerves entering the medulla near the CMJ (virgin control); (*b*) large and small nerves (arrowhead) crossing the CMJ (d 3 post partum). A, yellow autofluorescent cell (marking CMJ); C, cortex; M, medulla. Bar, 50 μm.

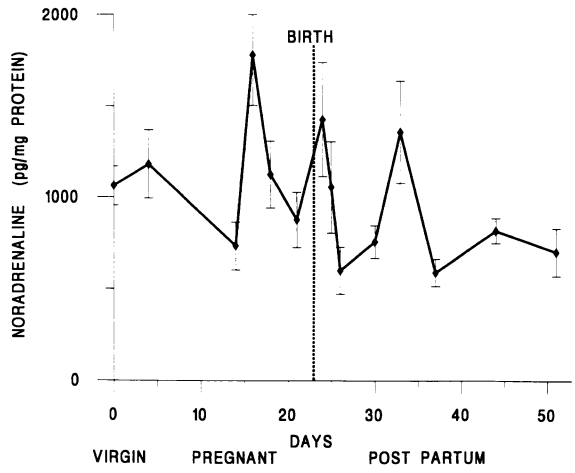

Fig. 8. Mean (±S.E.) NA levels (pg/mg protein) in the thymus of rats sampled during pregnancy and the post partum period.

blood vessels, but run independently, may be related to the fact that β-adrenoreceptors have been identified on thymocytes (Singh, 1979; Singh et al. 1979). Stimulation with catecholamines or the use of β-adrenergic antagonists alters the proportions of stem cells expressing Thy-1 and TL antigens, suggesting a role for NA in thymocyte development (Singh & Owen, 1976). Felten & Felten (1989) have suggested that NA interacts with receptors on the surface of many cell types in both primary and secondary lymphoid organs to affect proliferation, differentiation, and effector functions, including lymphocyte interactions in the immune response.

It has been suggested that sex steroids can modulate the numbers of catecholamine receptors in the uterus (Krall et al. 1978). The action of sex steroids on the thymus is well established (reviewed by Kendall & Clarke, 1994). It has not been previously proposed that they could act through adrenergic receptors, but this possibility must be considered.

This study shows that there are variations in both visualised nerves and neurotransmitter content in the thymus during the course of pregnancy and the post partum period. This suggests that the dramatic thymic changes that take place at these times are influenced both by central events (levels of catecholamines in peripheral blood) as well as local events mediated by transmitter changes in nerves.

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