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Identification of neural profiles containing vasoactive intestinal polypeptide, acetylcholinesterase and catecholamines in the rat thymus

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INTRODUCTION

The thymus gland and other lymphoid tissues are well known to receive a sympathetic noradrenergic nerve supply (Felten *et al.* 1985). Noradrenergic fibres enter the gland mainly in nerve bundles and plexuses around major blood vessels that run to the corticomedullary junction.

Nerves enter the cortex, travel into the cortex from subcapsular plexuses around the vasculature and branch into the cortex itself. In addition, there is a subcapsular plexus from which nerves run to the mid- and deep-cortex.

Acetylcholinesterase-positive profiles have been demonstrated in the thymus by several workers, distributed with blood vessels and branching mainly into the cortex and at the corticomedullary junction (Bulloch & Pomerantz, 1984). By combining an enzymatic method for acetylcholinesterase (AChE) with Prussian blue dye injection into blood vessels, we have demonstrated apparently cholinergic nerves (AChE-positive fibres) accompanying blood vessels in the connective tissue septa and into the gland as well as nerves branching unaccompanied into the cortex and medulla (Al-Shawaf, Zaidi & Kendall, 1987). However, studies on other tissues, especially brain, have investigated the problems of ascertaining that such profiles were in fact cholinergic nerve fibres and have concluded that acetylcholinesterase may not always be localised in neural profiles (Felten & Felten, 1988). In addition, other known cholinergic neurons fail to be demonstrated by AChE or monoclonal antibodies raised to choline acetyltransferase (ChAT). However, there is evidence to suggest that noradrenaline (NA) and AChE may be colocalised in some fibres (Burnstock, 1978).

Whatever the misgivings some workers may have regarding the use of AChE staining as an indicator for cholinergic innervation, the method is still regarded by many as an important indicator of cholinergic nerves.

In this study, immunohistochemistry with polyclonal antibodies against Protein Gene Product 9·5 (PGP 9·5) and Vasoactive Intestinal Peptide (VIP) were used, as were sucrose-phosphate-glyoxylic acid fluorescence (SPG) (de la Torre, 1979) and AChE histochemistry (Karnovsky & Roots, 1964) on thymus glands from CSE rats in order to assess the extent and character of thymic innervation. The sympatholytic agent 6-hydroxydopamine (6-OHDA) was used in some rats to ascertain the effect of sympathectomy (Thoenen & Tranzer, 1973; Kostrzewa & Jacobovitz, 1974) on total thymic innervation.

PGP 9·5 is a general cytoplasmic neuron-specific protein, structurally and immunologically distinct from neuron specific enolase (NSE) (Gulbenkian, Wharton & Polak, 1987). VIP, on the other hand, is present in peripheral nerves mainly in postganglionic cholinergic neurons innervating exocrine gland tissues such as the submandibular salivary gland, nasal mucosa, tongue and bronchi. It is a 28 amino acid peptide that was first described as a vasodilator substance in lung tissue (O'Dorisio, Wood & O'Dorisio, 1985). VIP-like immunoreactivity and high (AChE) activity have been observed in the same cells (Lundberg *et al.* 1980). Furthermore, the fact that VIP-like immunoreactivity is present in practically all principal ganglion cells of a clasically cholinergic ganglion, such as the sphenopalatine ganglion, further supports the hypothesis that VIP is present in many cholinergic neurons (Lundberg *et al.* 1980), where it appears to act as a neurotransmitter.

VIP is also synthesised and stored in immunoeffector cells and there are specific functional VIP receptors on human lymphocytes. Possible roles for VIP in the immune system include the modulation of lymphocyte migration (O'Dorisio *et al.* 1985) and modulation (mainly inhibition) of T cell responses to mitogenic stimulation (Ottaway & Greenberg, 1984).

In this study we present evidence for both catecholaminergic and cholinergic innervation of the thymus gland.

MATERIALS AND METHODS

Thirty male CSE rats in the 240–280 gm weight range were used to assess the extent of VIP-like and PGP 9·5-like immunoreactivity and SPG-positive and AChE-positive nerve profiles in the normal, untreated thymus. A further 30 adult CSE rats were used for the 6-OHDA experiments: 15 rats were injected with 0·2 ml 6-OHDA (80 mg/kg) in isotonic saline intraperitoneally while the remainder were injected with 0·2 ml saline vehicle. Four 6-OHDA-injected animals were killed on each of days 2, 4 and 8 post-injection, whilst the last group of three 6-OHDA-injected rats was given a further injection of 180 mg/kg 6-OHDA i.p at 10 days after the first injection and killed 24 hours later. The animals were killed by chloroform inhalation and the thymuses dissected out. Thymuses were then either divided into two parts, one for immunohistochemistry and one for SPG histochemistry, or processed as a whole for AChE histochemistry.

Immunohistochemistry

The specimens to be stained with VIP and PGP 9·5 were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7·4 for 24 hours, then washed in 20 % sucrose in PBS for 72 hours before being frozen on chucks in isopentane cooled in liquid nitrogen and cut in a cryostat at 6 μm. The sections were collected on gelatin/chrome alum-subbed slides and air-dried. Before staining, the slides were rehydrated in PBS for 15 minutes.

Polyclonal VIP antibody (Immuno Nuclear Corp., USA), and Polyclonal PGP 9·5 antibody (Ultraclone, UK) were then applied at various test dilutions. A dilution of 1:500 was optimal for VIP and 1:800 was optimal for PGP 9·5. The antibodies were diluted in PBS containing 0·1 % sodium azide, 1 % bovine serum albumin, 0·1 % triton and 0·1 % dl-lysine and applied to sections for 16 hours at room temperature. The sections were then washed for 15 minutes in three changes of PBS. A second layer of goat antirabbit IgG conjugated to FITC (Dako) was applied at a dilution of 1:80 for 90 minutes. The sections were washed in three changes of PBS and mounted in

glycerol/PBS mountant (Citifluor, UK) and observed with a fluorescence microscope with appropriate filters for FITC fluorescence.

The antibody against PGP 9·5 is raised against an unidentified human neuronal protein (Thompson *et al.* 1983) consequently, the term 'PGP-like' has been used throughout to describe the observed staining. The antibody against VIP cross-reacts slightly with secretin but not with other related peptides (manufacturer's specifications). Therefore, again, the term 'VIP-like' was used to describe the observed staining.

SPG fluorescence histochemistry

Specimens were immediately frozen and sections, cut in a cryostat at 20 μm, were picked up on gelatin/chrome alum-subbed slides, and immediately dipped three times (one second each) in a glyoxylic acid solution (100 ml distilled water, 10·2 g sucrose, 4·8 g KH_2PO_4 and 1·5 g glyoxylic acid, at pH 7·4) (de la Torre, 1979). Sections were dried in an air current. A drop of paraffin was applied to each section, and slides were put in an oven preheated to 90 °C for 2·5 minutes. The sections were mounted in paraffin and observed with a fluorescence microscope equipped with specific filters for catecholamine fluorescence.

AChE histochemistry

Thymuses were fixed in 4 % paraformaldehyde for 90 minutes, washed in PBS, then washed in 20 % sucrose/PBS for 24 hours. The thymuses were then frozen in isopentane cooled in liquid nitrogen, cut in a cryostat at 20 μm and sections were collected on coverslips. These sections were divided into four batches, three of which were pre-incubated with one of the following inhibitors for one hour: *iso*-OMPA (Tetraisopropyl-pyrophosphoramide) (Sigma),10^{-5} M, a non-specific cholinesterase inhibitor; physostigmine (Eserine) (Sigma), 10^{-5} M, a total cholinesterase inhibitor; BW.284C51 (Burroughs Wellcome), 10^{-6} M, a specific inhibitor (Austin & Berry, 1953). The fourth batch was pre-incubated in PBS.

All sections were washed in water before being incubated for 30 minutes at 37 °C in a phosphate buffer solution containing: 0·1 M-KH_2PO_4 and 0·1 M-Na_2HPO_4 at pH 6·0, 0·1 M sodium citrate, 30 mM copper sulfate and 5 mM potassium ferricyanide. Sections were then washed, dehydrated in a series of alcohols, cleared and mounted in DPX. AChE-positive structures were stained brown.

RESULTS

VIP, PGP and SPG

There were no differences in the distribution of nerves or their staining reactions between uninjected and control rat thymuses. PGP-like nerve profiles were seen accompanying major blood vessels as they passed through the perithymic adipose tissue to reach each lobe, and with blood vessels in the trabeculae taking blood to the corticomedullary junction (Fig. 1*a*). The nerves were arranged as a plexus in the tunica adventitia of the larger vessels (Fig. 1*b*). PGP-like varicose fibres branched off from these major nerve bundles and entered the cortex between the Type I epithelial cells limiting the gland. Blood vessels did not accompany these nerves. The nerves formed a richly branching plexus in the subcapsular cortex, that extended 10–20 μm into the cortex. The plexus was present around most of the lobules and extended along the trabeculae towards the corticomedullary junction where it sometimes formed an additional plexus. Some positive fibres penetrated deeper into the cortex and

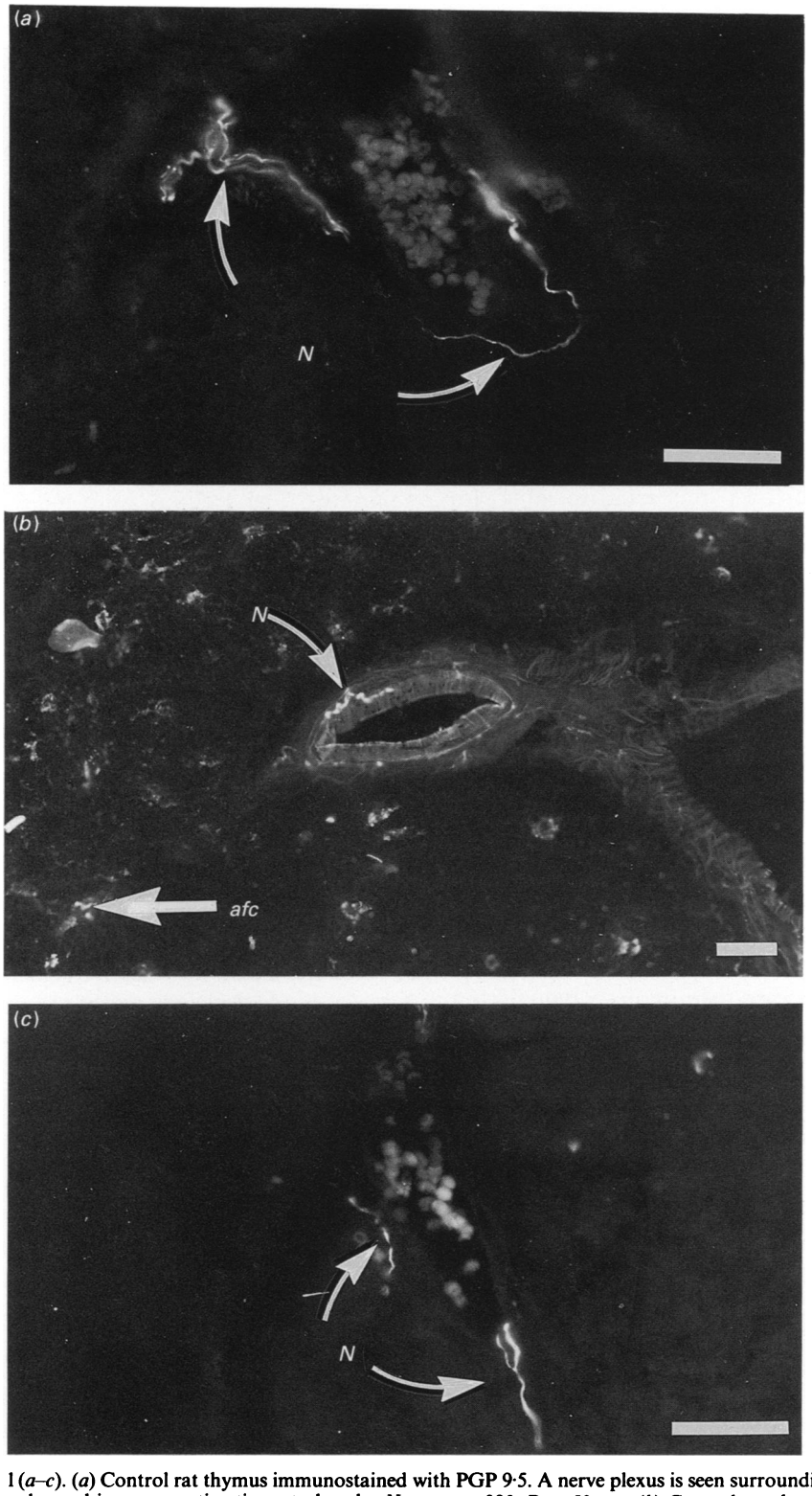


Fig. 1 (*a–c*). (*a*) Control rat thymus immunostained with PGP 9·5. A nerve plexus is seen surrounding a blood vessel in a connective tissue trabecula. *N*, nerve. × 320. Bar, 50 μm. (*b*) Control rat thymus immunostained with PGP 9·5. A nerve fibre is seen in the adventitia of a blood vessel in the thymus cortex. Autofluorescent cortical cells are seen in the vicinity. *N*, nerve; *afc*, autofluorescent cells. × 80. Bar, 100 μm. (*c*) Control rat thymus immunostained with VIP, showing nerve fibres in a blood vessel wall, with branches in the thymus cortex. *N*, nerves. × 320. Bar, 50 μm.

branched, possibly terminating near groups of large autofluorescent cells. No PGP-like fibres were observed in the medulla.

VIP-like nerves followed a similar distribution but their density was not so great. Individual nerve fibres were also varicose, but the cortical fibres particularly appeared to be finer than the majority of PGP-like nerves (Fig. 1*c*).

In the sections prepared for an examination of the catecholaminergic innervation using the SPG method, the innervation appeared more dense than the staining for PGP 9·5 due to the use of thicker sections, but the distribution of the nerves followed that described above for PGP-like nerves. Large arteries (600 μm in diameter) had as many as 15–20 nerve bundles in the tunica adventitia (Fig. 2*a*), and smaller nerves could be observed branching off to enter the cortex, without any accompanying blood vessels (Fig. 2*b*,*c*).

Two days after injection of 6-OHDA, a great reduction was seen in PGP-like nerve profiles. There were no positively staining fibres entering the gland with blood vessels or branching out from them. Some PGP-like nerves could be seen in the subcapsular area, although with greatly reduced density (Fig. 3*a*). A few PGP-like fibres, of much smaller diameter than those predominant in non-6-OHDA-injected animals were seen in the cortex and in connective tissue trabeculae. A greater density of PGP-like innervation was seen in sections from animals killed four days after injection of 6-OHDA compared with sections from animals killed after two days and the nerve density was further increased in sections from animals killed eight days after injection of 6-OHDA, probably indicating nerve regeneration (Fig. 3*b*). Fibres were particularly dense in the connective tissue trabeculae and subcapsular areas. Reappearance of nerve profiles occurred, by the fourth day post-injection, first in the major branches accompanying blood vessels and also in the subcapsular plexuses.

The reappearance of nerve profiles in the other areas of the cortex and in the corticomedullary junctional areas was not noticed until the eighth day post-injection. After a further injection of 6-OHDA (Day 11 post-primary injection), the reduction of positive neural profiles was broadly similar to that seen for specimens at two days post-injection.

VIP-like nerve profiles were seen to be of a smaller diameter than PGP-like nerves and were seen throughout the cortex. Individual nerves were sometimes seen in the subcapsular areas, but not forming plexuses like those of PGP-like nerves. Some VIP-like nerve profiles were also noted in the trabeculae in association with blood vessels. No VIP-like nerve profiles were seen in the thymic medulla. The injection of 6-OHDA appeared to have no effect on the density or staining patterns of VIP-like nerve fibres (Fig. 3*c*). Catecholamine-fluorescent nerve profiles were seen to enter the gland in control animals in thick bundles accompanying blood vessels. The nerve plexuses followed the vessels in the trabecular septa, branching out with branches of the blood vessels and also giving off branches to the cortex. Some fibres were seen to end in the environs of cortical autofluorescent cells. Subcapsular nerve plexuses were noted. No catecholaminergic nerve profiles were seen in the medulla.

There was total loss of catecholamine staining on specimens from animals killed two days post-6-OHDA injection. On Days 4 and 8 post-6-OHDA injection, a gradual increase of nerves showing positive staining was seen, which was broadly similar to that observed above for PGP-like profiles (although there was no complete obliteration of PGP-like positivity in 6-OHDA-treated animals). By four days after 6-OHDA, large SPG-positive nerve profiles accompanying blood vessels were noted, as were subcapsular nerve plexuses, and by the eighth day the full cortical and cortico-medullary junctional networks started to reappear (Fig. 3*d*). In specimens killed 24

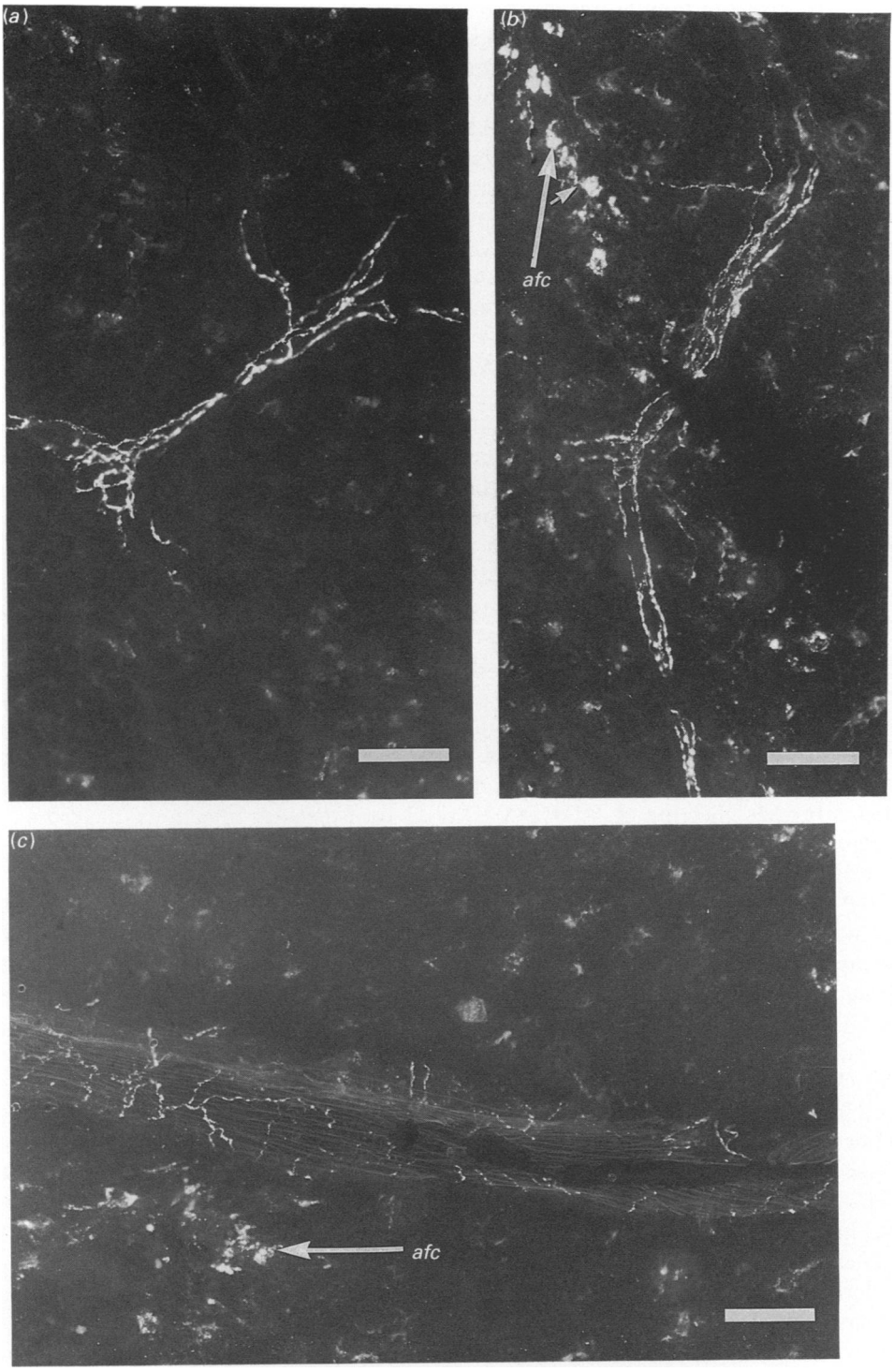


Fig. 2(*a–c*). (*a*) SPG (catecholamine)-fluorescent adrenergic nerves in a control rat thymus. A nerve fibre net is seen in a blood vessel wall in the thymus cortex. × 128. Bar, 100 μm. (*b*) SPG (catecholamine)-fluorescent adrenergic nerve fibres in a blood vessel wall of a control rat thymus. Nerve fibres are seen entering the thymus cortex, with autofluorescent cortical cells in the vicinity. *afc*, autofluorescent cells. × 128. Bar, 100 μm. (*c*) SPG (catecholamine)-fluorescent adrenergic nerve fibres seen branching from a vascular nerve plexus and being distributed in the thymus cortex of a control rat. *afc*, autofluorescent cells. × 128. Bar, 100 μm.

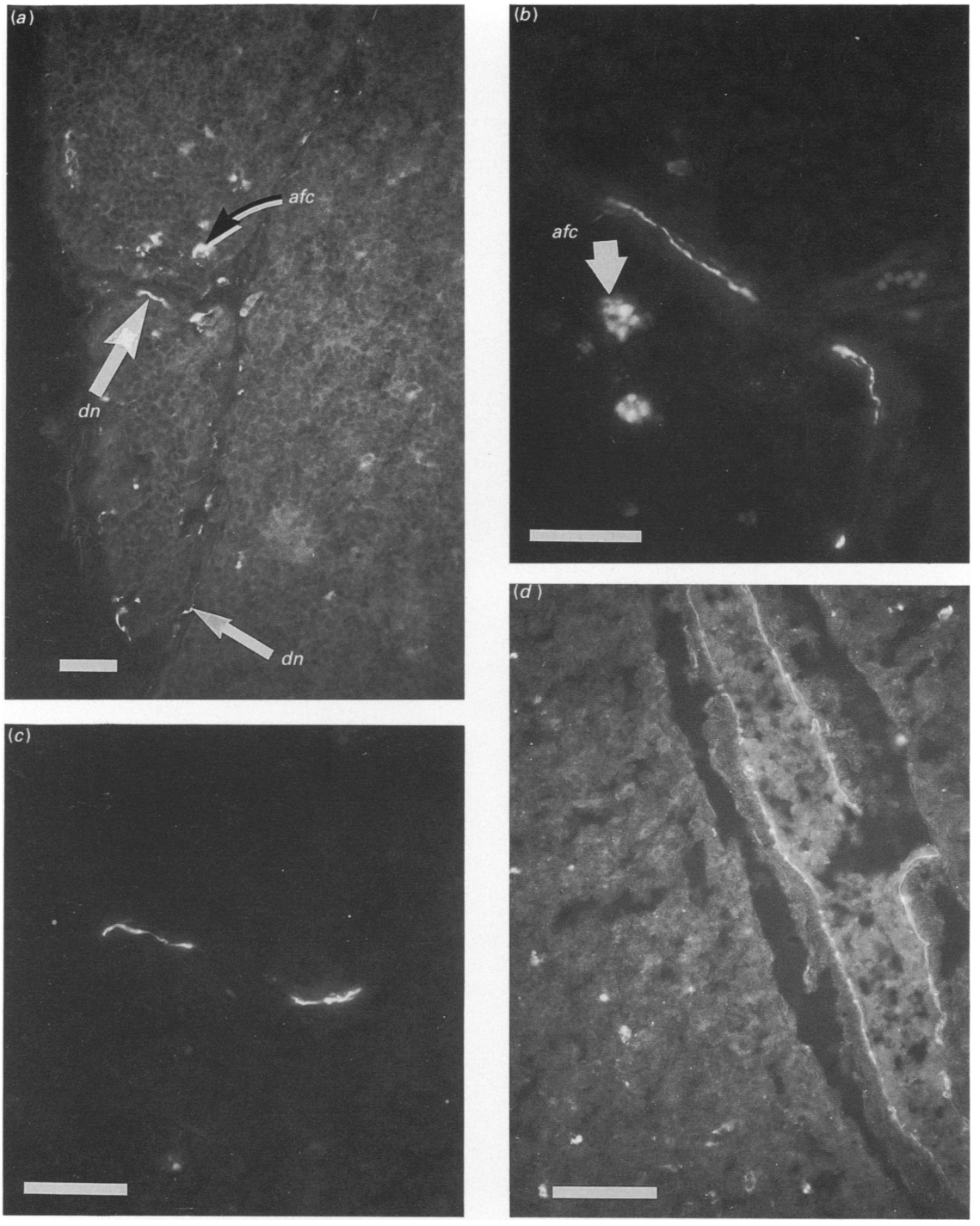


Fig 3 (*a–d*). (*a*) PGP-immunostaining in a rat thymus two days after 6-OHDA sympathectomy. Very few degenerated nerve profiles are seen in the thymus subcapsular area. *dn*, degenerated nerve; *afc*, autofluorescent cells. × 80. Bar, 100 μm. (*b*) PGP-immunostained rat thymus eight days after 6-OHDA sympathectomy, showing reappearance of nerve profiles in thymus cortex. *afc*, autofluorescent cells. × 320. Bar, 50 μm. (*c*) VIP-immunostained rat thymus two days after 6-OHDA sympathectomy. Nerve fibres in the thymus cortex appear to be unaffected. × 320. Bar, 50 μm. (*d*) SPG (catecholamine) fluorescence in a rat thymus eight days after 6-OHDA sympathectomy. Reappearance of nerve fibres in the thymus cortex is seen, surrounding a blood vessel. × 320. Bar, 50 μm.

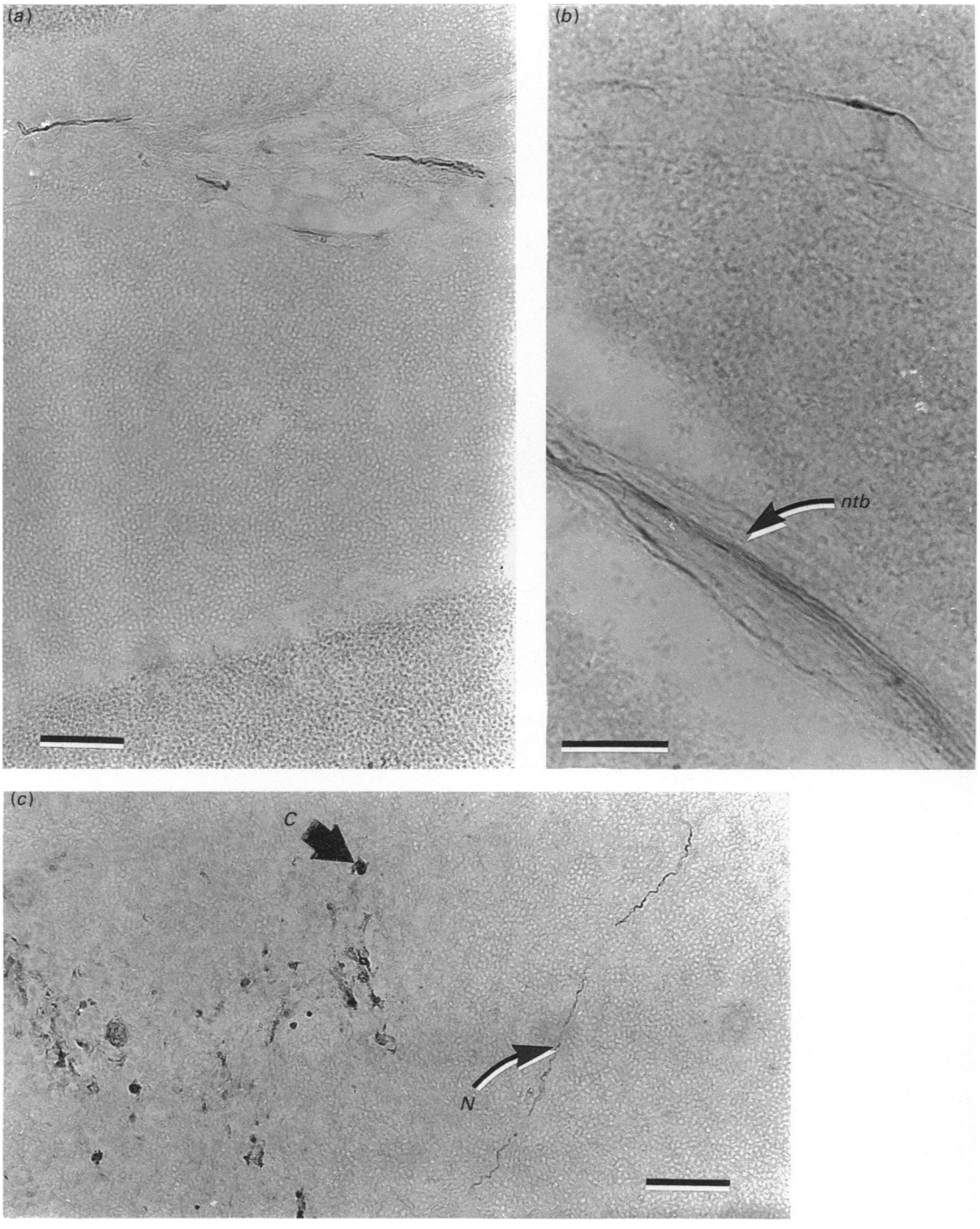


Fig. 4(*a–c*). (*a*) AChE histochemical staining on a control unsympathectomised rat thymus, with iso-OMPA inhibition. Nerve fibres are seen with blood vessels in the thymus cortex. × 128. Bar, 100 μm. (*b*) AChE histochemical staining on a control unsympathectomised rat thymus, with iso-OMPA inhibition. Nerve fibres are seen in the vicinity of blood vessels in a thymus connective tissue septum. *ntb*, large non-terminal nerve bundle. × 320. Bar, 50 μm. (*c*) AChE histochemical staining on a control unsympathectomised rat thymus, with iso-OMPA inhibition. AChE-positive cells are seen in the thymus medulla. A nerve (with a blood vessel) is seen in the vicinity. *N*, nerve; *C*, cell. × 128. Bar, 100 μm.

hours after a second injection (11 days after the first injection), no SPG nerve profiles were detected.

AChE

Sections of normal thymus stained for AChE showed networks of AChE-positive staining profiles entering the parenchyma of the gland with major blood vessels in the connective tissue septa. Thick positively-staining bundles of fibres were seen with large blood vessels (Fig. 4*a*, *b*). These followed the branchings of the blood vessels and fibres were also distributed to the medulla with small blood vessels. In the cortex, the main areas of concentration of the positively-staining fibres were in the subcapsular areas and the corticomedullary junctional areas. The density of fibres in the latter region was far greater than that in other areas of the cortex and in the medulla. AChE-positive profiles were also distributed around the blood vessels in the connective tissue septa.

AChE-positive cells were mainly grouped in the medulla. They were round or fusiform in shape and some had long processes. Fine AChE fibres were observed in their vicinity (Fig. 4*c*).

In 6-OHDA treated specimens stained for AChE, there was no difference in the distribution of the fibres, although there may have been a slight diminution in the overall density of AChE-positive profiles (Fig. 5*a–c*). AChE-positive cells were also unaffected by 6-OHDA injection. Sections pre-incubated in *iso*-OMPA (tetra-isopropyl-pyrophosphoramide) were similar to controls, while those pre-incubated in eserine and in BW.284C51 showed total lack of positive AChE staining.

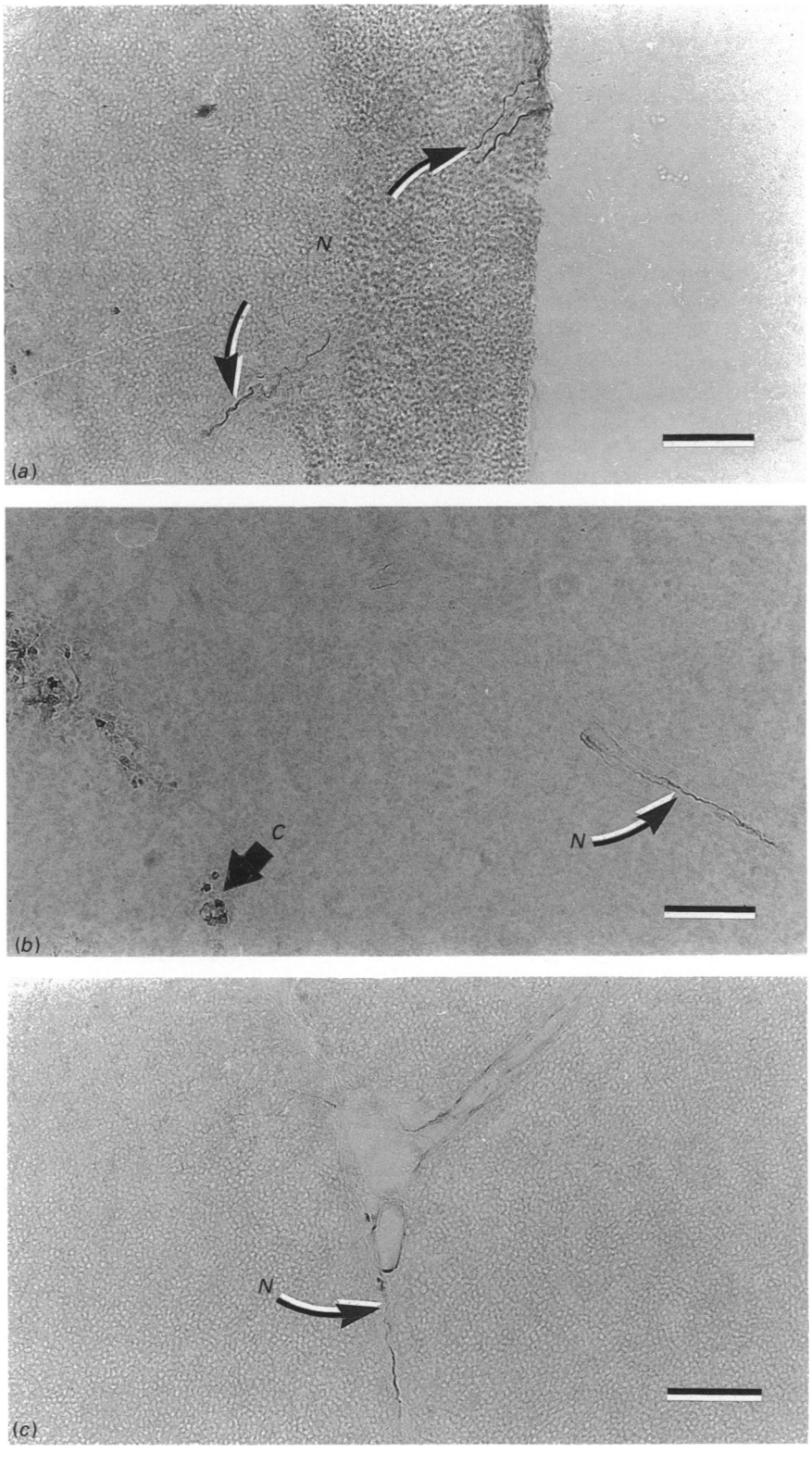
DISCUSSION

Central influence over the thymus (and the immune system in general) is expressed through the neuroendocrine system and the autonomic nervous system. Almost all pituitary hormones and hormones of pituitary target organs have been described as having neuroendocrine effects on immune functions (Berczi, 1986; Berczi & Kovacs, 1987) and interact with the thymus gland (Kendall, 1988). Autonomic innervation of many lymphoid organs translate central signals into neurotransmitter signals which influence immune functions through immune system cell receptors (Felten *et al.* 1987).

The nerves supplying the thymus gland are derived from the vagus, the phrenic, and the recurrent laryngeal nerves as well as from fibres originating in the stellate and other ganglia of the thoracic sympathetic chain (Bulloch & Pomerantz, 1984). The early presence of neural elements of the autonomic nervous system in thymic ontogeny was first described by Hammar (1935). Vagal fibres penetrated the embryonic thymus and infiltrated areas of parenchyma that defined the boundaries between the cortex and the medulla. Hammar also observed that fibres from the phrenic nerve penetrated the organ so that full innervation was acquired well before birth. Noradrenergic innervation of the thymus is well established (Felten *et al.* 1985, 1987; Felten & Felten, 1988).

The use of the polyclonal antibody PGP 9·5 and SPG fluorescence in our study confirmed these observations and showed that nerves enter the gland with the major blood vessels, form subcapsular nerve plexuses and accompany larger blood vessels to the corticomedullary junction. PGP 9·5, VIP and SPG staining (as well as previous work with AChE: Kendall, Al-Shawaf & Zaidi, 1988) also demonstrate nerve fibres leaving the main nerve bundles to penetrate the cortex.

Thymus glands from rats recently injected with 6-OHDA to destroy the sympathetic innervation had very few SPG-positive fibres but the VIP-like staining was unaffected. This persistence of VIP reactivity after 6-OHDA injection, together with evidence


N
(a)
C
N
(b)
N
(c)

from AChE histochemistry, suggests that the distribution of AChE-positive fibres in the thymus (around major blood vessels, in the subcapsular cortex, in the connective tissue septa, as well as individually in the medulla) resembles the distribution of VIP-like profiles (with the exception of the medullary element). This suggests a cholinergic component in thymic innervation where VIP is colocalised in cholinergic autonomic nerves in the same way as in other cholinergic systems (Lundberg *et al.* 1980). The fact that sympathectomy did not affect either AChE or VIP immunoreactivity further supports this. It has been suggested that VIP forms part of the neuro-immune axis. Specific receptors for VIP have been demonstrated on lymphocytes (O'Dorisio *et al.* 1985).

Although AChE is a component of cholinergic nerves, it has also been associated with other neurotransmitters (Lehmann & Fibiger, 1979; Lockridge, 1982). Nevertheless, it is considered (Bulloch & Moore, 1981) to be a marker for cholinergic innervation of the thymus because many of the vagal nerve fibres that innervate the thymus originate in cholinergic brainstem nuclei (Kasa & Silver, 1969; Contreras, Gomez & Norgren, 1980; Kalia & Mesulam, 1980; Bulloch & Moore, 1981). The possibility should not be discounted, however, that this enzyme is present in nerves terminating in lymphoid tissues, the principal transmitters of which are not acetylcholine (Bulloch & Moore, 1981). However, the short incubation times and controls used in this experiment suggest relatively high levels of specific cholinesterase that are normally characteristic of cholinergic nerves. Nance, Hopkins & Bieger (1987), using retrograde tracing of small injections of wheatgerm agglutinin-horseradish peroxidase into the thymus and into surrounding mediastinal and cervical structures, concluded that all labelled cells in the brainstem and cervical spinal cord observed following tracer injections into the thymus can be accounted for by spread of the tracer into surrounding structures. Injection of HRP into the trachea and longus colli muscle and other structures in the region of the thymus resulted in brainstem staining similar to that previously ascribed to thymus projections. The same authors were also sceptical of AChE staining in the thymus on the grounds that there was poor localisation to specific structures and that background staining was excessive. The short incubation times used in this study, together with optimum inhibition of non-specific cholinesterases enabled us to overcome these two problems. Horseradish peroxidase injection into the brainstem has also failed to reveal any anterograde labelling of nerves in the thymus (Felten & Felten, 1988). Thus the origin of cholinergic nerves in the thymus remains an area of continued controversy.

The effect of the autonomic nervous system on immune functions has been studied by Felten *et al.* (1985, 1987). They showed that chemical sympathectomy using 6-OHDA caused a marked reduction in both primary and secondary immune responses, an increase in B-lymphocyte proliferation, a diminished delayed-type hypersensitivity response and reduced cytotoxic T-cell activity. Similar functional studies have not yet been undertaken on the thymus gland. Several actions of an autonomic innervation have been postulated. Bulloch & Moore (1981) also suggest that early innervation of the murine thymus may be an essential part of its appropriate differentiation.

Fig. 5(*a–c*). (*a*) AChE histochemical staining in a rat thymus two days after 6-OHDA sympathectomy, with iso-OMPA inhibition of non-specific staining. Nerve fibres in thymus cortex, unaffected by sympathectomy. *N*, nerves. × 128. Bar, 100 μm. (*b*) AChE histochemical staining in a rat thymus two days after 6-OHDA sympathectomy, with iso-OMPA inhibition of non-specific staining. Nerve fibres, unaffected by sympathectomy, are seen with blood vessels. A group of AChE-positive cells is seen in the thymus medulla. *N*, nerve; *C*, cells. × 128. Bar, 100 μm. (*c*) AChE histochemical staining in a rat thymus eight days after 6-OHDA sympathectomy, with iso-OMPA inhibition of non-specific staining. Nerve fibres are seen in the thymus subcapsular area. *N*, nerve. × 128. Bar, 100 μm.

Ottaway & Greenberg (1984) and Ottaway (1984) concluded that local concentrations of VIP within tissues innervated by VIP-containing neurons may be sufficient to produce significant receptor occupancy and perhaps alter the function of T-cells present in the vicinity of such nerves, and that the interaction of T-cells with VIP could be an important aspect of the local regulation of mucosal immunological function.

The distribution of nerve fibres found in this study requires specific mention. The subcapsular and medullary epithelial cells are both secretors of thymic hormones and VIP and ACh that colocalise in exocrine glands are involved in peptide secretion. ACh affects mainly secretion and VIP mainly vasodilation (Lundberg *et al.* 1980). Whilst a control over secretion could also apply to the medulla where the vasculature permeates the stroma, the subcapsular cortex lacks blood vessels (other than in the capsule) and has no AChE positivity. Perhaps the two nerve nets have different functions due to the microenvironmental differences between them.

Lastly, the two nerve network sites are both areas where cells enter and leave the thymus and the autonomic nervous system could be involved in these phenomena. Non-pathological accumulations of lymphocytes around nerves are observed in the thorax of wild type mice. There is, in the nude mouse, an increased number of such lymphocyte accumulations, which is interpreted as representing an attempt by the nervous system to compensate for the lack of a competent thymus. The dense innervation observed in these areas might selectively regulate the passage of cells across this barrier.

SUMMARY

Sympathetic and parasympathetic innervation of the rat thymus is described using immunohistochemical, fluorescence histochemical and histochemical methods. Sympathetic innervation was found to enter the gland with the vasculature and to be distributed mainly in the subcapsular and corticomedullary junctional areas of the cortex. The parasympathetic innervation was also found to enter the gland with the vasculature, but was distributed to both cortex and medulla. Acetylcholinesterase-positive staining cells were seen in the medulla.

Ideas about the function of thymus innervation are discussed.

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