The Thymus as a Neuroendocrine Organ

Synthesis of Vasopressin and Oxytocin in Human Thymic Epithelium[a]

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INTRODUCTION

By 1985 arguments from the literature could be advanced to provide a rational basis for a neuroendocrine function of the thymus. First, embryologic studies revealed that cells of ectodermal origin, derived from the cephalic neural crest, migrated to

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the primitive thymic endodermal rudiment; they participated mainly in the formation of thymic mesenchyme, but a portion of thymic epithelium in fowl seemed to be neural-crest-derived.[1] Second, distinct epithelial cell populations were identified in the thymus through the use of the monoclonal antibody A2B5, which was found to recognize a complex ganglioside expressed on the cell surface of neurons, and neural crest-derived and neuropeptide-secreting endocrine cells.[2,3] Some A2B5-reactive cells were also found to contain thymic hormones, such as thymopoietin and thymosin-α_1.[1] Finally, Ott and Scott had reported in 1910 that extracts of thymic organs induced milk ejection in the goat,[4] an action that is actually known to be mediated by the neurohypophyseal peptide, oxytocin. To our knowledge, an intrathymic synthesis of neuropeptides has not been systemically investigated until this time.

The neurohypophyseal hormones, oxytocin and vasopressin, are produced in magnocellular neurons of the hypothalamic supraoptic and paraventricular nuclei. They are synthesized as large molecular weight precursors which are then cleaved during axonal transport into the nonapeptides, oxytocin and vasopressin, and their associated "carrier" proteins, the neurophysins.[5,6] Vasopressin and oxytocin are widely distributed in the central nervous system[7] and in peripheral organs such as ovary, testis, and adrenal.[8–10] Peripheral synthesis of oxytocin has been confirmed in bovine corpus luteum[11] and granulosa cells.[12]

We have recently reported that high amounts of oxytocin and neurophysin were detected by specific radioimmunoassays (RIAs) in the human thymus.[13] Thymic oxytocin was further characterized by gel filtration and high-performance liquid chromatography. Biological uterotonic activity of thymus-extracted oxytocin was also tested on isolated rat uterus; a characteristic uterine contraction occurred with an estimated oxytocin-like bioactivity in close quantitative agreement with the amount detected by RIA. Thymic contents of oxytocin and neurophysin were far greater than those expected from their known circulating levels, and declined with increasing age. Moreover, the molar ratio of oxytocin to neurophysin in the human thymus was similar to that found in the hypothalamoneurohypophyseal system. These findings strongly suggested a local synthesis of oxytocin and introduced the concept of a neuroendocrine function for the thymic gland.

In this paper, we present further data confirming the intrathymic synthesis of oxytocin and vasopressin as well as preliminary results about the distribution of oxytocin- and vasopressin-containing cells in the human thymus.

MATERIAL AND METHODS

Procurement of Human Thymus Glands

Thymus fragments were obtained from two young patients undergoing corrective cardiovascular surgery for congenital cardiopathies: one specimen was excised from a 4-year-old girl with atrial septal defect and another from a 1-year-old boy with persistent patent ductus arteriosus. Immediately after dissection, fragments for messenger ribonucleic acid (mRNA) analysis were frozen in liquid nitrogen and stored at −70°C. Fragments for immunohistochemistry were embedded in Tissue-tek and frozen at −70°C.

Analysis of Thymic mRNA

Poly(A)$^+$RNA was prepared from human thymus by extraction in guanidinium isothiocyanate followed by CsCl centrifugation; the resulting RNA was enriched for poly(A)-containing molecules by two passages over an oligo(dT)-cellulose affinity column.[14] 5 μg of this poly(A)$^+$RNA was spotted onto nitrocellulose filters and hybridized at 60°C overnight[11] with 10^6 cpm/ml of a specific ^{32}P-labeled fragment from either the human hypothalamic vasopressin cDNA or the human hypothalamic oxytocin cDNA.[14] These hormone-specific 3′ fragments, which correspond approximately to the third exons of the respective genes (FIGURE 1), were radiolabeled with ^{32}P by nick-translation to a specific activity of 5×10^8 cpm/μg. After stringent washing, the dot blots were exposed to X-ray film for 1 to 3 days and the resulting autoradiograms quantitated by scanning densitometry. Positive controls were provided by poly(A)$^+$RNA from human hypothalamus, negative controls by human putamen poly(A)$^+$RNA (both tissues courtesy of Dr. Piers Emson, Cambridge), as well as by poly(A)-depleted RNA from bovine cerebellum and muscle and by calf thymus tRNA.

Immunohistochemistry

Antiserum antineurophysin was raised in rabbit against purified bovine neurophysin; it was equally influenced by various preparations of different animal and human purified neurophysins, and so it detected oxytocin-associated as well as vasopressin-associated neurophysins, as previously described.[15] Antiserum antioxytocin was raised in rabbit against synthetic oxytocin coupled to bovine thyroglobulin.[12] It shared an uncomplete cross-reaction (± 40%) with arginine-vasotocin; cross-reactions with other peptides were: vasopressin, 0.3%; bovine oxytocin-associated neurophysin, 0.4%; oxytocin terminal tripeptide, 1.25%; thymopoietin, thymopentin, and thymulin,

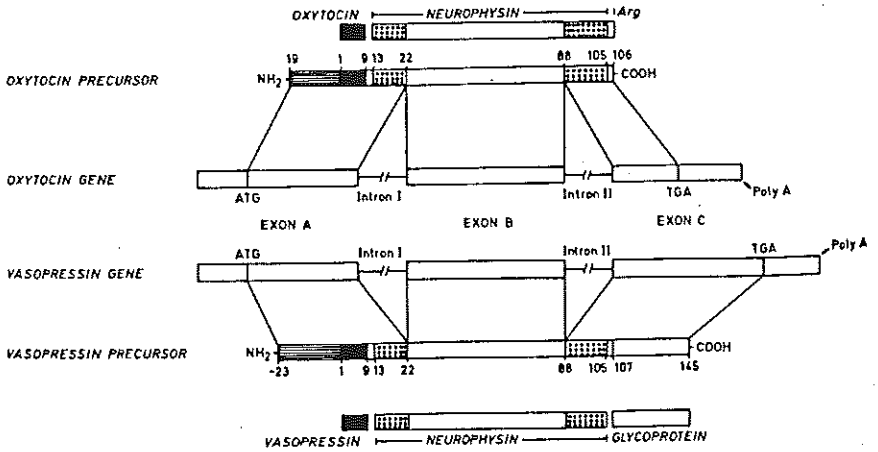

FIGURE 1. Structures of the oxytocin and vasopressin genes and precursors. (Adapted from Ivell and Richter.[33])

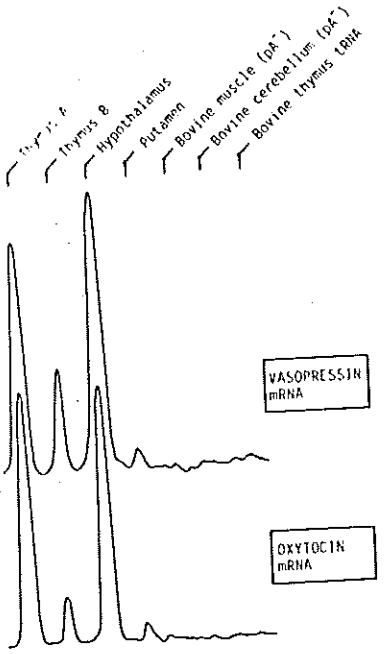

FIGURE 2. Dot blot analysis of poly(A)$^+$RNA from human thymuses. 5 μg of each human or bovine RNA, as indicated, was subjected to dot hybridization as described in the MATERIAL AND METHODS section. The resulting autoradiograms were scanned densitometrically and expressed by an arbitrary ordinate relative only to the hypothalamic sample used as internal standard.

< 0.01%. Antiserum antivasopressin was purchased from UCB-Bioproducts (Brussels, Belgium). It showed an incomplete cross-reaction with oxytocin (± 50%), and so it was preincubated with 5 μg of synthetic oxytocin before immunostaining.

Five-micron frozen sections of thymic fragments were cut on a cryostat and collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium potassium sulfate. Tissues were fixed for 1 min in cold acetone. Before incubation with antisera, the sections were soaked in Dulbecco's phosphate-buffered saline solution supplemented with 1% fetal calf serum and 0.05% Tween-20, for 30 min to remove the embedding medium. Sections were immunostained with the alkaline phosphatase-antialkaline phosphatase technique (APAAP).[16] Incubations with the different antisera were performed overnight at 4°C. Dilutions of antineurophysin, vasopressin, and oxytocin antisera were 1:200, 1:1000 and 1:1000, respectively. Specificity of binding was controlled by the inhibition of immunoreactivity obtained after a preincubation of the antisera with their respective antigen. Control experiments were also performed with normal rabbit serum as primary antibody step.

RESULTS

Analysis of Thymic mRNA

Dot blot analysis of the poly(A)$^+$RNA from the two thymuses indicates the presence of both vasopressin and oxytocin mRNAs (FIGURE 2). The radiolabeled

probes were derived from the 3′ regions of the hypothalamic cDNAs, which show less than 20% mutual nucleotide homology,[14] and in controls (not shown) exhibited absolutely no cross-reactivity. In the thymus from the 4-year-old girl (FIG. 2, thymus A) both oxytocin and vasopressin mRNAs were present in concentrations equivalent to those found in adult male hypothalamus. The thymus from the younger boy (FIG. 2, thymus B) exhibited somewhat less of both mRNAs, but levels were still significantly above background.

Immunohistochemistry

The reactivity pattern of antineurophysin serum with a normal human thymus (4-year-old girl) is presented in FIGURE 3A. Two immunoreactive regions were clearly identified: a narrow band in the subcapsular cortex (SCC) and distinct stromal cells in the medulla (M). The cortex (C) was negative in its greater part. FIGURE 3B shows that thymic reticuloepithelial cells and Hassall's corpuscles (HC) in the medulla were recognized by antineurophysin antiserum. The periphery—but not the keratinized center—of Hassall's corpuscles (HC) was strongly positive (FIG. 4). Similar data were obtained with both antivasopressin and antioxytocin (not shown). TABLE I summarizes the distribution of neurophysin-, vasopressin- and oxytocin-containing epithelial cells in the human thymus. The comparison with the distribution reported for A2B5-reactive epithelial cells[3] reveals a close parallel between the localizations of A2B5-positive and neuropeptide-producing cells.

DISCUSSION

We have already reported the presence in the human thymus of a peptide sharing immunologic, physicochemical, and biological properties with authentic oxytocin.[13] The coexistence of almost equimolar amounts of neurophysin strongly suggested, but did not prove, an intrathymic synthesis of oxytocin by cleavage from a common precursor as demonstrated in the hypothalamus[6] and in the bovine corpus luteum.[11] The detection of mRNA for oxytocin and vasopressin in high quantities establishes that their genes are actively expressed in the young human thymus and that the presence of oxytocin and vasopressin in this tissue must be attributed to local synthesis and not to an accumulation of the systemically circulating pituitary hormones. To our knowledge, this is the first demonstration of a neuropeptide synthesis in the thymus. Moreover, calculation of relative amounts shows that the human thymus can produce levels of oxytocin and vasopressin mRNAs comparable to those in the adult male hypothalamus. This observation is of considerable interest with regard to a possible physiological role of thymic vasopressin and oxytocin.

Immunohistochemical studies reveal that the thymic neuroendocrine cells share the same distribution with A2B5-reactive cells, mainly in the subcapsular cortex and the medulla. This parallelism, as well as the significance of A2B5 recognition,[2] strongly suggests that at least a part of A2B5-positive cells can be vasopressin- or oxytocin-secreting cells. If this hypothesis were confirmed by double-immunostaining studies, it would raise the question of the embryonic origin of these cells and the possibility of their neural crest origin.

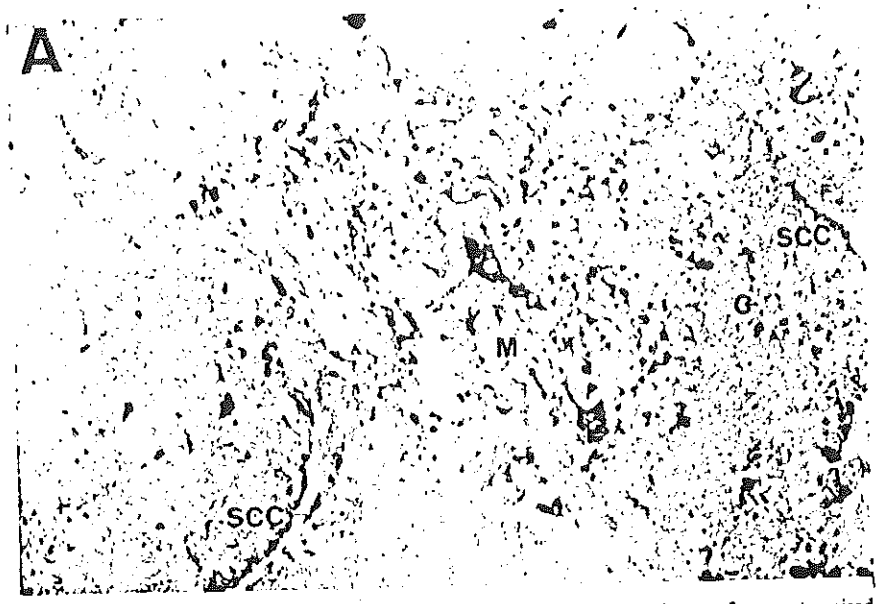

FIGURE 3A. General distribution of neurophysin-secreting cells in a thymus fragment excised from a 4-year-old girl. Immunoreactive cells are identified in the subcapsular cortex (SCC) and in the medulla (M). The cortex (C) is negative in its greater part. (Magnification × 100.)

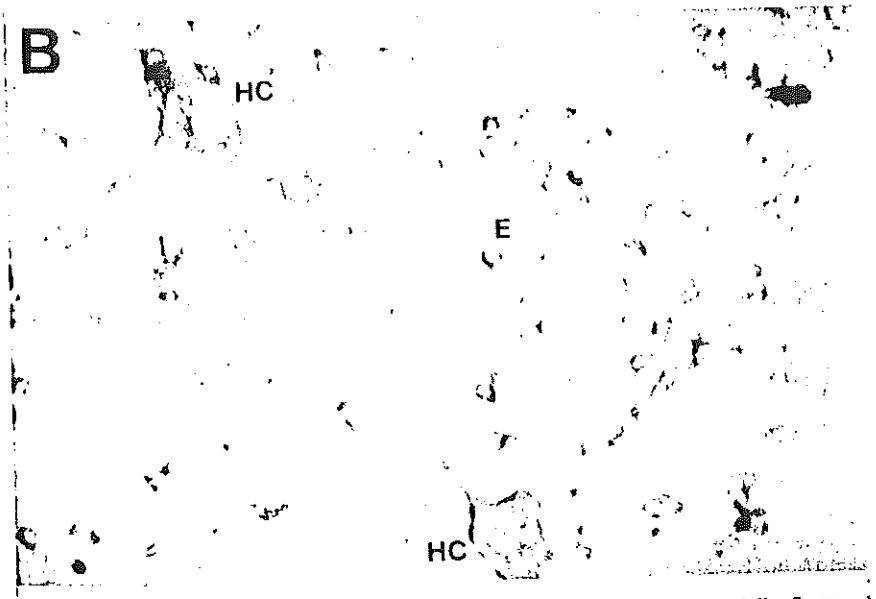

FIGURE 3B. Characterization of neurophysin-immunoreactive cells in the medulla. Scattered stromal cells (E) and two Hassall's corpuscles (HC) are immunostained. (Magnification × 400.)

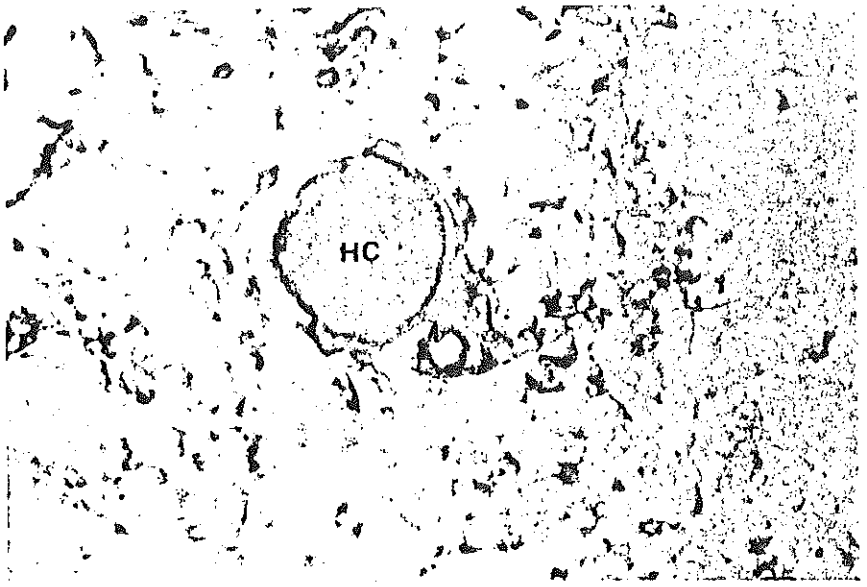

FIGURE 4. The periphery of Hassall's corpuscles, but not the keratinized center, is neurophysin-immunoreactive. (Magnification × 400.)

The discovery of an intrathymic synthesis of vasopressin and oxytocin automatically poses the question as to its physiological meaning. Although thymic vasopressin and oxytocin may participate in the general functions described for these peptides (i.e., homeostasis of water metabolism for vasopressin; induction of uterine contractions and milk ejection for oxytocin), it is tempting to speculate that they could exert intrathymic paracrine actions on thymocytes. In this context, both vasopressin and oxytocin were shown to replace interleukin-2 (the T-cell growth factor) for gamma-interferon production by mouse splenocytes.[17] We have recently confirmed this action on cultured human peripheral lymphocytes (unpublished data). Conversely, treatment of cancerous patients with interleukin-2 was reported to induce a marked fluid retention,[18] and this could be due in part to an enhancement of vasopressin production by interleukin-2 or to an interaction of interleukin-2 at the vasopressin receptor site. Thus, thymic vasopressin and oxytocin could have "interleukin-2-like" properties, and this could be of significant importance with regard to the recent observation of interleukin-2 receptor expression as a differentiation marker on intrathymic stem cells.[19,20] Vasopressin was also reported to stimulate DNA synthesis in bone marrow cells[21] and in chondrocytes.[22] Oxytocin was shown to stimulate glucose oxydation in rat thymocytes.[23] Therefore, some co-mitogenic, inductive, or repressive actions of vasopressin and oxytocin during lymphocyte differentiation and/or proliferation are attractive hypotheses to consider in future investigations.

Another possibility would be that thymic vasopressin and oxytocin exert some control on lymphocyte hormonal productions. Activated lymphocytes are able to

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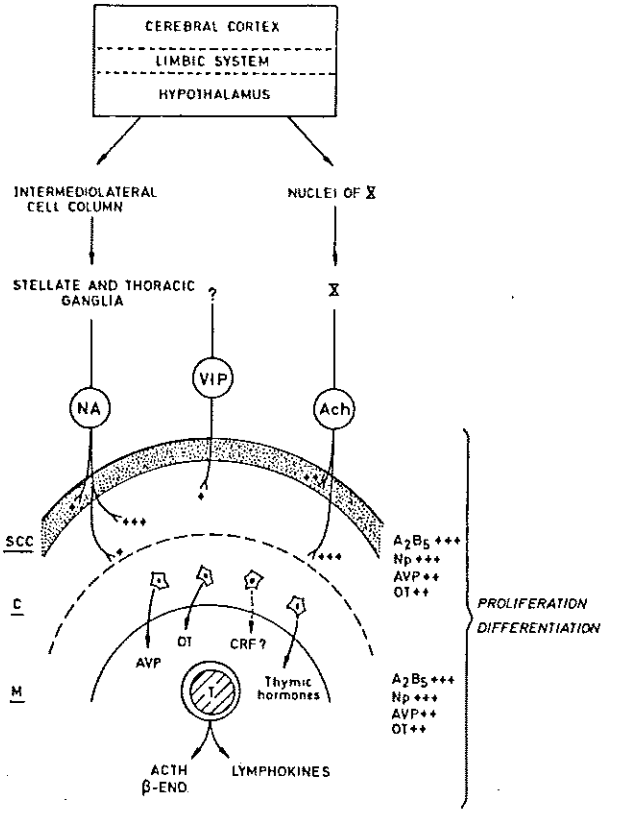

FIGURE 5. The neuroendocrine thymus-lymphoid axis; see explanation in the DISCUSSION section. Thymic neuroendocrine cells are mainly distributed in SCC and M, but only medullary cells have been figured. The presence of CRF-secreting neuroendocrine cells is hypothetic, but plausible because of the coexpression of CRF within oxytocin- and vasopressin-immunoreactive hypothalamic neurons.[25] The relative distribution of autonomous innervation is also indicated. Np = neurophysin; AVP = vasopressin; OT = oxytocin; NA = noradrenaline; Ach = acetylcholine; VIP = vasoactive intestinal peptide.

TABLE 1. Distribution of Neurophysin-, Vasopressin- and Oxytocin-Producing Cells in the Human Thymus Compared with That Reported for A2B5-Reactive Cells[3]

Structure	Neurophysin	Vasopressin	Oxytocin	A2B5
Subcapsular cortex	+++	++	++	+++
Inner cortex	0	0	0	0
Medulla	+++	++	++	+++
Hassall's corpuscles	+++	+	+	+
Interstitium	0	0	0	0

NOTE: Number of crosses is based on the number of positive cells in each region and the intensity of staining.

produce adrenocorticotropin (ACTH) and β-endorphin (β-END),[24] two polypeptide also coordinately synthesized in the anterior pituitary from a large-molecular-weight precursor. At the hypothalamohypophyseal level, vasopressin and oxytocin are co-localized with corticotropin-releasing factor (CRF)-secreting neurons.[25] Vasopressin and oxytocin were shown to modulate the action of CRF on antehypophyseal ACTH secretion: in rats, both vasopressin and oxytocin potentiate the inductive effect of CRF on ACTH production,[26] whereas in humans oxytocin seems to inhibit ACTH secretion and, secondarily, the adrenal production of cortisol.[27,28] Recently, Smith *et al.* reported that vasopressin enhanced the number of lymphocytes containing immunoreactive ACTH in the presence of CRF.[29] So, the regulatory mechanisms of ACTH secretion in the anterior pituitary could be transposed to the lymphocyte level. If this view was further confirmed, a model of neuroendocrine thymus-lymphoid axis could be proposed (FIG. 5). The thymus is directly connected with the central nervous structures by parasympathetic fibers originating from vagal nuclei in the brainstem, and by orthosympathetic fibers from the stellate and other ganglia of the thoracic chain.[30] Acetylcholinesterase-positive fibers are mainly found in the subcapsular cortex and the corticomedullary junction.[30] Noradrenergic fibers enter with arterial and subcapsular plexuses and project throughout the thymic cortical parenchyme, including the corticomedullary boundary.[31] Some vasoactive intestinal peptide (VIP)-like immunoreactive fibers from unknown origin are found in the deep region of the thymic cortex.[32] Like other neurosecretory cells, thymic neuroendocrine cells could translate a neural input to a neuropeptide secretion. Thymic neuropeptides would then exert some influence on T-lymphocyte differentiation or proliferation and could modulate lymphocyte productions of lymphokines (such as gamma-interferon) or hormones (such as ACTH and β-END). The exact significance of this neuroendocrine thymus-lymphoid axis and its intervention in stress-induced physiopathological states remain to be defined in the future.

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