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The Neurohormonal Thymic Microenvironment: Immunocytochemical Evidence that Thymic Nurse Cells Are Neuroendocrine Cells

(with 2 color plates)

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Abstract. Thymic neuroendocrine cells were identified by immunofluorescence in the murine thymus through the use of monoclonal antibody A2B5, and specific polyclonal antisera against neurophysin (NP), oxytocin (OT) and arginine vasopressin (AVP). Two reactive regions were clearly identified: the subcapsular cortex and the medulla. A close correspondence was observed between A2B5-reactive and NP-immunoreactive cells in the medulla. An important epithelial population of the subcapsular cortex, the thymic nurse cells (TNCs), were found to be A2B5-positive and to contain immunoreactive NP, OT and AVP. The neuroendocrine nature of TNCs was further substantiated by their high reactivity with an antiserum against neuron-specific enolase. These observations demonstrate the presence in the thymus gland of an original neuroendocrine microenvironment which could be of functional importance in the mediation of central influences upon T lymphocyte differentiation.

The thymus is the major site of T cell differentiation from their hematopoietic precursors [5, 15]. This complex process of maturation requires direct cell-to-cell receptor-mediated interactions, as well as paracrine information via cytokines and secretory products of thymic epithelial cells, the thymic hormones [2, 13]. The thymic nurse cells (TNCs) provide one striking example of very close interactions between developing T cells and thymic epithelial components [20, 26, 27]. First isolated from mouse thymus after enzymatic digestion, TNCs consist of outer-cortical epithelial cells which enclose a large number of actively dividing thymic lymphocytes [26, 27]. TNCs have also been described in situ on thymic sections obtained from different species [6, 23, 29]. The expression by TNCs of major histocompatibility complex (MHC) determinants [7, 27], and the immunocompetence, although limited, of TNC-derived thymocytes [1] support the concept that this specialized microenvironment plays an important role in T cell education/selection processes.

The neurohypophyseal peptides oxytocin (OT) and arginine vasopressin (AVP) might also intervene in T cell differentiation. We have recently described the presence in the human thymus of a peptide sharing immunological, biological and physicochemical properties with the neurohormone OT [11]. Immunoreactive (ir) AVP has also been extracted from rat and mouse thymuses [22]. OT and AVP are structurally similar nonapeptides which are mainly synthesized in the hypothalamic supraoptic and paraventricular nuclei as the N-terminus of larger precursor proteins [18]. During their axonal transport towards the neurohypophysis, these precursors are enzymatically cleaved into the active nonapeptides OT and AVP, and their respective associated neurophysins (NP) I and II [3]. Intrathymic coexistence of almost equimolar concentrations of ir OT and NP strongly suggested a local synthesis of these neuropeptides which was confirmed by the detection of OT and AVP messenger RNAs in human thymic extracts [12]. Immunocytochemistry with the alkaline phosphatase-antialkaline phosphatase technique revealed that ir NP, OT and AVP-containing cells were mainly distributed in the subcapsular cortex and the medulla of the human thymus [12].

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In this paper, we demonstrate by immunofluorescence cytochemistry the presence of ir neurohypophyseal peptides in the subcapsular cortex and the medulla of murine thymus. Further characterization of positive cells was achieved through the use of the monoclonal antibodyA2B5 which binds to a complex ganglioside expressed on the membrane of neuronal, glial, and neuroendocrine peptide-secreting cells [8, 9], as well as on some subsets of thymic hormone-producing epithelial cells [14]. Since TNCs constitute a major cellular component of the subcapsular epithelium [16, 23], we judged of high interest to investigate also their neuroendocrine nature.

Material and Methods

The thymuses used in these studies were excised from 1-month-old C57BL/Ka mice. TNCs were isolated by serial sedimentations after enzymatic digestion as previously described [16]. The thymic fragments and the pellets of TNCs were embedded in a cryoprotectant (Tissue-Tek; Miles Laboratories), and frozen at –70 °C. Five micron sections were cut with a cryostat at –20 °C, dried for 10 min under ventilated cold air, and fixed for 1 min in cold acetone. Forty thymuses at least were needed to constitute one pellet of TNCs, and six different pellets were examined in this study.

The antiserum against purified bovine NP was raised in our laboratory, and recognizes the central constant region of NPs; therefore, it detects OT-associated as well as AVP-associated NPs [21]. Cross-reactivity is less than 0.5% with prolactin, growth hormone and adrenocorticotropin; less than 0.01% with OT, AVP, folliculo-stimulating hormone, luteinizing hormone, thymopoietin, thymopentin and thymulin.

Polyclonal antisera against OT and AVP were provided by UCB Bioproducts (Belgium). Since anti-AVP cross-reacts partially with OT, it was preincubated overnight with $5 \times 10^{-6} M$ OT, as indicated before [12]. These antisera do not cross-react with other known neuropeptides or pituitary polypeptides.

A2B5 mouse hybridoma was obtained from American Type Culture Collection (ATCC HB-29), and was recultured in our laboratory.

The antiserum against bovine γ/γ enolase – or neuron-specific enolase (NSE) – was purchased from Dakopatts (Belgium). Cross-reactivity is absent with α subunits (glial), and very weak with β subunits (muscle).

Sections were incubated for 30 min at room temperature with anti-NSE (1:200), for 3 h with crude A2B5 hybridoma supernatant, and for 18 h at 4 °C with anti-OT (1:1,000), anti-AVP (1:1,000), and anti-NP (1:200). Antibodies were visualized with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (for A2B5) or anti-rabbit Ig (for others) and, in double immunofluorescence studies, with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit Ig (Nordic Laboratories).

Control experiments included incubations with normal rabbit or mouse serum as first-step antibodies, as well as preincubations of antisera with their homologous antigens (synthetic OT and AVP, both at $5 \times 10^{-6} M$.

Results and Discussion

The distribution of A2B5 and NP immunoreactivities in the murine thymus is presented in figure 1. Two reactive zones were clearly identified: the subcapsular cortex (fig. 1a, b), and the medulla (fig. 1c, d). A similar distribution was found for ir OT and AVP (data not shown). Double immunofluorescence studies revealed a close correspondence between medullary A2B5-positive and ir NP-containing cells (fig. 1c, d). A2B5-negative cells which were labelled by anti-NP were extremely rare. This distribution was identical with that previously reported for A2B5, as well as for thymopoietin and thymosin-α_1 [14]. The localization of ir OT and ir AVP-containing cells was also the same in the human thymus [12].

The colocalization of A2B5 and neuropeptides in the subcapsular cortex incited us to investigate the neuroendocrine feature of specialized lymphoepithelial complexes derived from that zone, the TNCs [16, 23, 27]. Double immunofluorescence studies revealed that the epithelial component of TNCs – but not the engulfed thymocytes – strongly reacted with A2B5 and anti-NP (fig. 2a, b), anti-OT (fig. 2c, d), or anti-AVP (fig. 2e). The majority of isolated TNCs were so double labelled by A2B5 and antineuropeptides but, actually, we do not know if some TNCs contain both OT and AVP. The neuroendocrine nature of the TNCs was further confirmed by their high reactivity with anti-NSE serum (fig. 2f). In control experiments, no significant immunostaining of TNCs (or thymic sections) could be observed with normal rabbit or mouse serum as first-step antibody (fig. 3a), or after preincubation of the specific antibody with its respective antigen (fig. 3b).

While the precise nature – epithelial or dendritic – of the neuroendocrine medullary cells remains to be defined, the conjunction of three different neuroendocrine markers (A2B5, neuropeptides and NSE) demonstrates that TNCs constitute a part of the diffuse neuroendocrine system. In this view, the TNCs represent an original, if not unique, example of an extremely intimate association between a neuroendocrine element and immune developing cells, the TNC-engulfed thymocytes. In itself, this observation greatly accredits the concept that the neuroendocrine system may exert an influence upon T cell differentiation. The involvement of TNCs in this process was initially suggested by the finding that TNCs expressed high levels of MHC class I and class II antigens which are known to intervene in self-recognition and selection of T cell repertoire [26, 27]. The differentiative capacity of the MHC-positive TNC microenvironment was recently examined by the expression of T cell differentiation markers on TNC thymocytes and their sensitivity to cortisone (which is related to the state of maturation [5]). The majority of TNC thymocytes were cortisone-sensitive and expressed an immature phenotype, but a minority of them were cortisone-resistant and expressed a

mature phenotype [7]. Other characteristics of TNC-associated thymocytes are their high mitotic rate [27], and the presence of interleukin-2-secreting among them [25].

Our findings now support that OT and AVP might constitute differentiative signals in the TNC microenvironment. Interestingly, a secretory activity of TNCs was early suggested by the observation of regular membrane invaginations [27], but the presence of typical secretory granules has not yet been reported. Previously, AVP was shown to be mitogenic for thymocytes [28] and bone marrow cells [17], while both AVP and OT can replace interleukin-2 requirement for gamma interferon production by mouse splenocytes [19]. More recently, AVP was reported to modulate CRF-induced adrenocorticotropin production by human leukocytes as AVP does at the adenohypophyseal level [24]. In parallel with our investigations, a rational model of a neuroendocrine thymo-lymphoid axis could be proposed. The thymus is directly connected with central nervous structures through autonomic innervation [4, 10]. Like other neurosecretory cells, thymic neuroendocrine cells could translate neural autonomic inputs into neuropeptide secretions. Thymic neuropeptides could then exert a local paracrine influence upon T cell differentiation/proliferation, but could also modulate lymphocyte productions of lymphokines (such as gamma interferon) or hormones (such as adrenocorticotropin or β-endorphin) [24]. Indeed, the exact configuration and significance of this axis remains to be further examined, but we believe that its action would be to mediate central influences upon the immune system.

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