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Review Article
Thymic Expression of Neuroendocrine Self-Peptide Precursors: Role in T Cell Survival and Self-Tolerance

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For a long time the thymus was considered to be an intrinsic component of the endocrine system but the endocrine model of cell-to-cell signalling failed to be fully validated in this organ. With the discovery of its primary role in T-lymphopoiesis, the endocrine role of the thymus progressively vanished from the literature, although the thymic influence was still believed to be mediated by a humoral mechanism (1). However, in the past 15 years, the question of a neuroendocrine component in thymic physiology has resurfaced. From a number of studies, it now appears that the thymus is a crucial site for cross-talk between the neuroendocrine and immune systems, especially during foetal development (2). Thymic epithelial cells (TEC) and nurse cells (TNC) express a repertoire of neuroendocrine-related genes/precursors, and thymic polypeptides may serve as signals interacting with receptors on developing pre-T lymphocytes. This cryptocrine form of cell-to-cell signalling could play a role in T cell development and maturation. In addition, there is ample evidence that thymic neuroendocrine-related polypeptides behave as self-antigens which are presented to pre-T cells, and could induce the negative selection of T cells bearing a randomly rearranged antigen receptor (TCR) orientated against endogenous neuroendocrine families (self-reactive T cells). The objective of this review is to expose most of the scientific arguments which support the important role of the thymus in the education of T lymphocytes to recognize and tolerate neuroendocrine functions.

The establishment of immunological self-tolerance

Two fundamental properties characterize the immune system: the presence of cellular and molecular elements which defend the host against a universe of infectious agents (non-self) and, simultaneously, its inability to react against its host. This latter property is called immunological self-tolerance. The induction of self-tolerance involves a cascade of mechanisms, from the early steps in immune cell ontogeny to an advanced stage in life (3). For the T lymphocyte system, the primary steps towards tolerance occur within the thymus. To complete their differentiative programme, immature T cells receive signals from the thymic cellular microenvironment. Such signals may be emitted by thymic stromal cells (like hormones or cytoldnes), or may result from direct interactions between cell adhesion molecules expressed on pre-T cells (thymocytes) and thymic stromal cells (4, 5). During differentiation, immature T cells randomly rearrange the genes coding for the segments of their TCR. Many of these random TCR combinations are orientated against self-antigens which are expressed in the thymic microenvironment, then presented by proteins encoded in the major histocompatibility complex (MHC). The interaction of self-reactive T cell clones with their cognate self-antigens is thought to lead to their negative selection, either by programmed cell death (apoptosis), or by developmental arrest. This process of thymic clonal deletion was demonstrated with the use of mouse mammary tumour virus (MMTV)-encoded superantigens (6), and with transgenic mice expressing a TCR specific for the male antigen (H-Y) (7). Since the thymus does not express all the components of the self-structure, this organ does not delete all potential autoreactive T cells. Consequently, the existence of other mechanisms for developing tolerance (such as T cell anergy) at the periphery was postulated, and they were effectively shown to intervene in the process of immunological self-tolerance. Nevertheless, thymic clonal deletion of self-reactive T cells is by far the most important mechanism involved in self-education of the immune system (8).

The paradox of thymus selection (T cell life) and self-tolerance induction (T cell death)

Self-peptides are not only involved in the induction of central T cell self-tolerance but also intervene in the process of T cell maturation and positive selection (9, 10). Thus, the thymus is the site for an important paradox of contemporary cell biology: How can T lymphocytes be both positively and negatively selected in the thymic microenvironment (11)? A first explana-

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tion proposed that TEC were responsible for T cell positive selection, whereas other thymic bone-marrow derived stromal cells (macrophages and dendritic/interdigitating cells) induced deletion of self-reactive T cell clones (12, 13). However, this hypothesis is not supported by recent experiments which have established that TEC also are able to delete self-reactive T cells (14). The 'avidity/affinity hypothesis' has been proposed as another explanation of the thymic paradox (15, 16). This hypothesis is based on experiments showing that lymphocytes bearing transgenic TCRs (specific for defined antigens) do not mature in organ cultures of foetal thymuses from MHC class I-defective animals. However, they do so if peptides related to the cognate antigen of their TCR are added in the cultures. So, T lymphocytes are positively selected if their TCR is barely engaged with self-peptide at low concentrations (10^{-12} M), and are deleted if TCR is strongly engaged with self-peptide at high concentrations (10^{-6} M). Since the affinity of a TCR for its cognate antigen is rather low (10^{-8} M at the maximum) (17), the intrathymic concentration of self-peptides is of crucial importance for determining positive or negative T cell selection. If the experiments mentioned above have convincingly shown that T cell selection is peptide-specific, and depends on ligand concentration, one may question the nature and the amount of peptide/MHC combinations that contribute *in vivo* to positive or negative selection of a particular TCR in a normal thymus. Because of their high polymorphism, thymic MHC proteins cannot establish the discrimination between self and nonself antigens. Given the hazardous nature of the recombination of TCR gene segments, an important question now is the precise identity of thymic peptides which are presented to developing T cells and which support T cell positive and negative selection (18).

Development of the thymic cellular microenvironment

The term 'thymus' is derived from the Greek word 'Θυμος', and means both 'courage' and 'thyme'. Galien suspected that the thymus could be the seat of courage and affection because of its vicinity close to the heart (19). The general shape of the thymus resembles the leaves of the thyme plant. The major part of the thymic parenchyme is constituted by an epithelium which expands from the endoderm of the third pharyngeal pouch on each side into the surrounding mesenchyme. During the expansion of the endoderm, some ectoderm is incorporated into the epithelial primordium (20, 21). Inclusion of ectodermal cells could explain the heterogeneity of TEC, but also the analogous immunophenotype of TEC from the medulla and the outer cortex of thymic lobules. The interactions of the epithelial rudiment with mesenchymal cells from the cephalic neural crest are absolutely necessary for a proper development of the thymus (22). Only after interactions have occurred between endodermal epithelium and mesenchymal cells derived from the neural crest is the primordial thymus competent to attract lymphoid stem cells and provide the epithelial microenvironment within which pre-T cells may proliferate and differentiate. This was shown by the important thymic developmental defects which follow ablation of the cephalic neural crest (22). Both in human conditions and in animal models, there are examples in which the defective thymic development is accompanied by immune deficiencies. The DiGeorge syndrome is characterized by the congenital absence of thymus, parathyroids, as well as defects in the heart and truncal vessels. It has been suggested that this syndrome results from the failure of the migration of the cephalic neural crest (22, 23). Mice in which the homeobox gene hox-1.5 was disrupted present thymic aplasia, parathyroid hypoplasia or aplasia, reduced thyroid tissue, and frequently associated defects in the heart and great arteries (24).

A particular population of TEC in the outer and subcapsular cortex are TNC (25). They are very large epithelial cells (with a diameter up to 50 μm) which contain a number of internalized thymocytes (immature or pre-T lymphocytes). The thymocytes in TNC are not phagocytosed, but are engulfed within caveoles delimited by TNC plasma membrane; this process is called 'emperipolesis'. Within TNC caveoles, thymocytes display a high mitotic index (26). Functionally, TNC may be involved in T-cell negative selection since TNC-derived cell line has been shown to induce *in-vitro* deletion of thymocytes bearing transgenic TCR (27). Ultrastructural analyses have confirmed that TNC are not only able to present antigens, but also possess the intracellular machinery for antigen processing (28).

The thymic stroma also includes cells derived from bone marrow: macrophages and dendritic cells. Macrophages are dispersed troughout thymic parenchyme, in the cortex, as well as in the medulla. Dendritic/interdigitating cells represent the main population that strongly expresses MHC class II proteins in the thymic medulla (29).

The expression of MHC class I and II molecules by thymic macrophages and dendritic cells is linked to their activity as professional antigen-presenting cells (APC).

The thymus has been long considered as a lymphoepithelial organ (30) and the lymphoid compartment forms a 'passenger' cell population of the thymus. Firstly from foetal liver then from bone marrow (31), T cell precursors are attracted and migrate within the thymus where they engage in different types of interactions with TEC and thymic stromal cells. Different forms of cell-to-cell signalling can be distinguished along the pathways of T cell differentiation, namely cell-to-cell adhesion and autocrine/paracrine signalling. However, with the exception of rare paraneoplastic syndromes leading to the oversecretion of thymic hormones into the bloodstream, the classic (neuro)-endocrine type of cell-to-cell signalling is not encountered within the thymus. From 100 T cell precursors which migrate into the thymic environment, about only 10 mature T lymphocytes will leave the lymphoid organ in a state of functional competence and self-tolerance. Thus, the thymus is primarily a 'graveyard' for self-reactive T lymphocytes.

Thymic neurohypophysial-related peptides

At the beginning of this century, Ott and Scott described the galactogogue activity of thymic extracts injected into the goat (32). At that time, oxytocin (OT) had not been identified as the primary mediator of galactokinesis and the oxytocic activity of thymic extracts was not further characterized. Ir-OT could be extracted by acetic acid from human thymuses and quantified by a specific RIA using antiserurn AS02 against OT (33). High performance liquid chromotography (HPLC) analysis showed a single peak of ir-OT eluting with

the same retention time as synthetic OT. On isolated rat uterus, thymic extracts induced oxytocic contraction and the bioactivity quantitatively concorded with the amounts detected by RIA. The galactogogue action of human foetal thymic extracts was also described (34). Ir-neurophysin, the 10 kDa protein associated in the structure of all neurohypophysial precursors, was also detected in the human thymus and analysed by G-75 gel filtration. The molar ratio of irOT (2.2–18.4 ng/g) to ir-neurophysins (24–142 ng/g) was similar to that found in the hypothalamo-neurohypophysial axis, suggesting a local synthesis from a common precursor. Positive dot blot hybridizations of human thymic mRNA with bovine OT and vasopressin (VP) cDNA probes provided another argument for *in-situ* synthesis (35). Independently, ir-VP was also detected in rat thymic extracts and thymic VP concentrations were shown to be regulated by steroids (36). The presence of ir-OT was confirmed by HPLC in the rat thymus and rat thymic ir-OT concentrations were modulated by different treatments, further supporting the intrathymic synthesis of an OT-like peptide (37). Contrary to the case in humans, the content of ir-OT in the rat thymus increases with ageing (from 80.4 ± 5.2 to 249.2 ± 13.5 pg/mg protein), while ir-VP concentration decreases (from 8.2 ± 0.7 to 1.75 ± 0.2 pg/mg protein) (38). Ir-VP was also detected in the human thymus but VP concentrations (0.01–0.06 ng/g) were much lower. Thus, at the peptide level, both in the human and in the rat thymus, OT is the dominant peptide of the neurohypophysial family. Nevertheless, using a 3′ RACE-PCR, both proOT and proVP genes were shown to be transcribed in the murine thymus (39). The hypothesis that the thymus could be the site of expression of another neurohypophysial-related gene (such as vasotocin) was also examined (40), but there is no evidence that the mammalian genome contains additional neurophysin-related genes (41).

By immunocytochemistry, TEC and TNC from different species were shown to express polypeptide precursors of the neurohypophysial family, and the use of specific mAbs against OT and VP revealed a dominance of the OT lineage (42, 43). In a transgenic rat model, an overexpression of VP was also detected in the epithelial compartment of the thymus (44). Thymic nurse cells constitute an intimate neuroendocrine-immune microenvironment since their epithelial component (but not the TNC-engulfed pre-T cells) contains neurohypophysial-related peptides and expresses the phenotype of neuroendocrine cell types (45). However, the synthesis of OT in TEC/TNC is not coupled with the secretion of the non-apeptide or its neurophysin in the supernatant of human TEC/TNC primary cultures. In the murine thymus, ir-OT is not located in secretory granules but is diffuse in the cytosol, in vesicles of the endoplasmic reticulum, and associated with keratin filaments (46). Interestingly, similar ultrastructural features were also reported for OT and VP expressed by murine spleen eosinophil-like cells (47).

As discussed above, the thymic function is closely associated with the presentation of the self-molecular structure to developing T cells. This action was long thought to be mediated by thymic macrophages and dendritic cells only, but there is now considerable evidence that TEC and TNC are actively involved in the induction of central self-tolerance (48, 49). Since OT and its associated neurophysin are coexpressed by TEC and TNC, we hypothesized a processing of thymic proOT that could be related to antigen presentation instead of classical neurosecretion.

Using affinity-chromatography with a monoclonal antibody (mAb) directed against the monomorphic part of human MHC class I molecules (50), we identified in TEC/TNC plasma membranes a 55-kDa protein which was labelled both by anti-MHC class I mAb and anti-neurophysin antibodies (51). Since anti-neurophysin antiserum does not crossreact neither with MHC class I proteins, nor with β2-microglobulin, this 55-kDa membrane protein may represent a hybrid protein including both a neurophysin domain (10 kDa) and a MHC class I heavy chain-related domain (45 kDa). The precise biochemical mechanisms under this hybrid neurohypophysial/MHC class I membrane protein remain to be deciphered. The origin of this protein could reside at the post-transcriptional level (such as a trans-splicing phenomenom) or at the post-translational level (such as the ATP-dependent binding of ubiquitin to protein targeted to proteolysis). Following this putative explanation, the MHC class I domain would be implicated in membrane targeting of this hybrid protein, whereas neurophysin binds OT for presentation to pre-T cells. Other authors have shown the translocation of a neurophysin-like material in the cell membranes of cancer cells, and have provided strong arguments supporting the behaviour of neurohypophysial-related peptides as candidate tumoral antigens (52, 53). Thus, both in the hypothalamo-neurohypophysial axis and in the thymus, the neurophysin part of the OT precursor fulfils the same function: binding of the active non-apeptide OT and tranport to the external limit of neurones or TEC/TNC. The tyrosine residue in position 2 of OT and VP plays an important role in their binding to neurophysin (54). Interestingly, the tyrosine residue in the same position plays a crucial role in the binding of antigens to some MHC class 1 alleles for their presentation (55). The particular features of thymic OT-mediated T cell education to the neurohypophysial family can be related to the observation of a dissociation between thymic T cell education to self and peripheral T cell recognition of antigens (56). Moreover, in addition to the presentation by MHC class I and II proteins, recent studies have revealed the existence of other mechanisms of antigen presentation by nonpolymorphic molecules (such as CD1 (57)).

The antigenic behaviour of thymic OT was further demonstrated by another type of experiments. The immunological recognition of OT by specific mAbs at the outer surface of human TEC plasma membrane induced a marked secretion of the cytokines interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF) in the supernatant of TEC cultures (58). Given the nature of the epitopes recognized by anti-OT mAbs, we were able to conclude that the molecule OT is fully processed at the level of the TEC plasma membrane. The absence of biological effects following the treatment of TEC cultures with anti-VP mAbs supports the hypothesis that thymic OT behaves as the self-antigen of the neurohypophysial hormone family.

Cryptocrine signalling in the thymus network

The model of cell-to-cell cryptocrine signalling has been proposed by Funder (59) to characterize the direct

membrane-to-membrane exchange of chemical informations between large epithelial nursing cells (like Sertoli cells in the testis or TNC in the thymus) and immature elements which migrate and differentiate at their contact (respectively, spermatids or thymocytes). Besides its role as a self-antigen, there is evidence that OT mediates a cryptocrine-type signalling between TEC and TNC and pre-T cells. Neurohypophysial peptide binding sites have been detected in the rat thymus and on rat thymocytes (60, 61), on a murine pre-T cell line (RL12-NP) (62) and on murine cytotoxic T cells (62, 63). The Kd of neurohypophysial peptide binding sites expressed by murine T cells was 0.15 ± 0.07 nM. In the rat thymus, oestrogens were shown to increase the affinity of OT binding sites from 0.199 ± 0.016 to 0.122 ± 0.008 nM (64). Interestingly, using specific molecular cDNA probes, the expression of the rat V 1b (or V3) receptor was identified in tissues outside the anterior pituitary, including the thymus (65). Very recently, using RT-PCR and specific probes, the expression of OT receptor was identified on mouse $CD3^-$ and $CD3^+$ thymocytes (66). The mitogenic effect of neurohypophysial peptides on rat thymocytes was first described in 1969 (67), while OT was reported to stimulate glucose oxidation by rat thymocytes (68). On the basis of antagonist effects, murine pre-T cells seem to express a Vlb (or V3) subtype of VP receptor, while mature cytotoxic T cells harbour receptors of the OT-type. This suggests that the neurohypophysial receptors expressed by T lymphocytes could 'mature' in parallel with their differentiation. In both T cell types, neurohypophysial peptide receptors transduce low concentrations of both OT and VP (1 nM) via the phosphoinositide pathway. Neurohypophysial-related signals (1 nM) increase the incorporation of tritiated thymidine by freshly isolated murine thymocytes, suggesting a mitogenic effet (62). The observations of numerous points of focal adhesion between OT-containing TEC and immature T cells (46) led us to investigate the hypothesis that neurohypophysial peptides could stimulate focal adhesion-related kinases in thymocytes. Western blots of RL_{12}-NP-extracted proteins with anti-phosphotyrosine revealed a number of proteins, the phosphorylation of which was stimulated either by OT or VP (both at 1 nM). Two of these proteins were precipitated with anti-focal adhesion (FAK) mAb 2A7; one was identified as $p125^{FAK}$ and the other as a coprecipitating 130-kDa protein (probably $p130^{Cas}$). Another protein phosphorylated by OT in RL12-NP cells was identified as paxillin, a 68-kDa protein located at focal adhesion sites and associated with $p125_{FAK}$. Oxytocin was more potent than VP in inducing $p125_{FAK}$ phosphorylation and this OT effect was inhibited by a V1 receptor antagonist, confirming that immature T cells bear V1-type VP receptors (69). Stimulation of focal adhesions could promote T cell interactions with the thymic cellular microenvironment which are fundamental for the T cell differentiation programme. Thus, the bulk of available data support the existence of a cryptocrine cell-to-cell signalling within the thymus that is mediated by neurohypophysial-related signals originating in TEC and TNC and their functional receptors expressed by developing T cells. Because the intrathymic ir-OT concentrations are in agreement with the high affinity Kd of neurohypophysial receptors expressed by pre-T cells, general physico-chemical considerations are met to make functional this intra-thymic signalling. This is not the case for the neurohormones OT and VP, due to their low blood concentration. The precise phenotype of the pre-T cell subsets which are expressing neurohypophysial receptors inside the thymus remains to be further defined.

The existence of a functional signalling between thymic OT and receptors expressed by immature and cytotoxic T cells raises the possibility of modulating T cell activity by OT receptor antagonists. In whole-blood cell cultures, OT hexapeptide antagonists inhibit the production of interleukin-1β (IL-1β) and IL-6 elicited by human T cell activation with anti-CD3 mAb (70). Specific antagonists of OT receptors expressed by immune cells could offer a therapeutic benefit in circumstances during which a relapse of severe devastating autoimmune diseases must be avoided (such as during *post partum* or during lactation).

Application to other neuroendocrine polypeptide families

A number of neuroendocrine-related polypeptides have been detected and characterized in TEC and thymic stromal cells from different species (Table 1). Neurotensin (NT) and somatostatin have been extracted from the chicken thymus, especially after hatching, and have been characterized both immunochemically and chromatographically (71). Recently, we have shown that ir-NT is expressed at the cell surface of human TEC. Cultured human TEC contain ± 5 ng ir-NT/10^6 cells, of which 5% is associated with plasma cell membranes. High performance liquid chromatography analysis of ir-NT present in human TEC revealed a major peak of ir-NT corresponding to intact NT1–13. Ir-NT was not detected in the supernatant of human TEC primary cultures. Using an affinity column prepared with an anti-MHC class I Ab, NT-related peptides were retained on the column and were eluted together with MHC class I proteins (72).

Neurokinin A (NKA) is the peptide of the tachykinin family encoded in human and rat TEC by the preprotachykinin A (PPT-A) gene (73). Thymic *PPT-A* expression appears to be glucocorticoid-dependent since adrenalectomy of Sprague-Dawley rats markedly enhances thymic expression of *PPT-A* (and *NPY*) mRNAs (Ericsson and Geenen, unpublished observations). Interestingly, NKA exerts IL-1-like mitogenic effects on murine thymocytes (74), suggesting that tachykinin receptors expressed by immature T cells could be implicated in an accessory pathway for T cell maturation and positive selection. The amino acid sequence of NKA shares the same C-terminal epitope with other members of the tachykinin family, and the leucine residue in position 9 could be used in the binding to some MHC class I alleles, thus making NKA the self-antigen of the tachykinin family. The other tachykinin encoded by *PPT-A*, substance P, is not detected in TEC but is present in sensory nerve fibres of the thymus (75). Thymic specific receptors for substance P are associated with the vasculature in the medulla, where they could control local blood flow and vascular permeability (76).

The expression in the rat thymus of natriuretic peptides (ANP, BNP and CNP) is well documented. ANP seems to be the dominant thymic peptide and is expressed by thymic macrophages, while ir-CNP is expressed by thymocytes. The different types of natriuretic peptide receptors were also

TABLE 1. The Repertoire of Neuroendocrine Peptides Expressed in the Thymus.

Neuroendocrine families	Physiological aspects	Thymic repertoire
Neurohypophysial peptides		
Vasopressin (VP or ADH)	Water metabolism Vasoconstriction	OT≫VP
Oxytocin (OT)	Reproduction (CNS and periphery)	
Insulin family		
Insulin	Glucose metabolism	IGF-II > IGF-I≫Insulin
Insulin-like growth factor I (IGF-I)	Growth control	
Insulin-like growth factor II (IGF-II)	Foetal development	
Parathormones		
Parathormone (PTH)	Calcium metabolism	PTH-rP≫PTH (135)
Parathormone-related peptide (PTH-rP)		
Calcitonins		
Calcitonin (CT)	Calcium metabolism	CGRP≫CT (136)
Calcitonin gene-related peptide (CGRP)		
Tachykinins		
Substance P (SP)	Pain-inflammation Mastocyte degranulation	NKA≫SP (NKB?)
Neurokinin A (NKA)		
Neurokinin B (NKB)		
TGF-β superfamily	Differentiation signals Immunosuppressive effects	TGF-β1, TGF-β2? (137)
Natriuretic peptides		
ANP	Sodium metabolism	ANP?
BNP		
CNP		
Neuromedins		
Neurotensin (NT)	Hypothermia Analgesia Pancreatic exosecretion	NT
Neuromedin N	Vasodilatation	

detected by RT-PCR (77, 78). Treatment of murine foetal thymic organ cultures (FTOC) with ANP has been shown to decrease the total thymocyte yield in FTOC and to increase the CD4−8− and to decrease the CD4+8+ thymocyte subpopulations (79).

A series of anterior pituitary hormone immunoreactivities have been detected in different TEC subpopulations of the human thymus. These TEC populations are separate from OT/VP/neurophysin-containing epithelial cells. However, it is not yet clear whether these hormones are locally synthesized or sequestered in TEC from peripheral blood (80), though they have also been identified in cultured rat thymic fragments (81).

Thymic expression of insulin-related genes

In 1965, on the basis that AKR female mice develop hypoglycaemia and thymic hyperplasia associated with lymphoid leukaemia, Pansky *et al.* reported the presence of an insulin-like peptide within the thymus of AKR strain mice, as well as of bovine and porcine species (82). Mouse thymic extracts induced a marked hypoglycaemia when injected into young female AKR mice. The biological action of thymic extracts even exceeded that of similarly prepared pancreatic extracts. In the line of our working model that central T cell tolerance of neuroendocrine functions is induced by the thymic repertoire of neuroendocrine self-antigens, a series of investigations were undertaken to identify the dominant member of the insulin family expressed in the thymic microenvironment. By immunocytochemistry with a panel of specific Abs directed against distinct epitopes of the insulin family, ir-IGF-2 was identified as the dominant member of the insulin family expressed by TEC/TNC (83). A mAb against proinsulin (the specific epitope of which is centred against the histidine residue at position 10 of the B chain) (84) revealed a slight labelling, but outside thymic lobules, in the interstitial tissue of the thymic capsule and in interlobular septae. Thymic labelling was also negative with mAbs against the C-terminal part of the insulin B chain. A few IGF-1 positive cells were stained in thymic lobules but they were not epithelial; their distribution and morphology were similar to those of macrophages. Of interest, murine macrophages were shown to express IGF-1 Ea and Eb transcripts (85). Interferon γ inhibits macrophage IGF–1 at the transcriptional level (86), whereas colony-stimulating factors induce IGF-1 mRNA (87). Ir-IGF-2 was not detected in the supernatant of human TEC primary cultures and, with the use of confocal microscopy, a large part of ir-IGF-2 was found to be associated

with the outer surface of TEC plasma membranes. This was not the case neither for IGF-1, nor for insulin. In the human thymus, IGF-2, IGF-1 and (pro)insulin concentrations were, respectively: 96.7 ± 10.6 ng/g, 42.9 ± 5.0 ng/g, and < 0.1 ng/g wet weight. *IGF2* transcripts have been isolated from whole human thymic extracts, as well as from primary cultures of human TEC. With RT-PCR and different specific primers, the expression of *IGF2* in the human thymus was found to be controlled by the same promoters as in other foetal and adult extrahepatic tissues (88, 89). The effects of *Igf2* overexpression under the control of the MHC H-2Kb promoter have been investigated by the generation of transgenic mice. The highest levels of transgene expression were found in thymus and spleen. Only the thymus showed a significant increase of weight in these transgenic mice, in agreement with the high mRNA expression within this organ (90).

Going back to the initial observations made by Pansky *et al*. there is now evidence that the thymic insulin-like reticular factor corresponds to IGF-2. The IGF-2 structure closely related to (pro)insulin explains a cross-reactivity with the polyclonal Abs directed against insulin that were used in 1965. The hypoglycaemic properties of IGF-2 have been well described (91) and might explain the biological activity of thymic extracts on glucose metabolism. Moreover, the syndrome of hypoglycaemia and lymphoid leukaemia associated with thymic hyperplasia of some AKR female mice could result from the overexpression of *Igf2* in hyperplastic thymic epithelium, with a subsequent secretion of IGF-2 in the bloodstream, and a profoundly disturbed thymic T cell lymphopoiesis.

The hypothesis of a central T cell tolerance of the insulin family and, secondarily, of the peripheral insulin-secreting pancreatic islet β cells was further supported by the observation that transcripts of proinsulin and of 67-kDa isoform of glutamic acid decarboxylase (GAD) genes can be detected in the murine thymus with 30 cycles of RT-PCR (92). Thymic insulin gene (*INS*) expression was highest in perinatal mice and persisted until 12 weeks of age. Two recent papers confirmed these findings and reported that *INS* transcripts, as well as (pro)insulin protein can be detected at very low levels (100–1000 fmol/g wet weight) in the human foetal thymus (93, 94). The question of an illegitimate *INS* trancription was ruled out by the RIA detection of ir-(pro)insulin within thymic tissues. Preliminary *in-situ* hybridization studies have shown *INS* transcripts within murine thymic dendritic cells, but presence of the protein could not be evidenced by immunocytochemistry (Homo-Delarche, personal communication).

Type 1 and type 2 IGF receptors have been detected on rat thymocytes and murine thymoma cells (95), on human phytohemagglutinin A (PHA)-activated T cells and on anti-CD3-activated human T lymphocytes (96, 97). Kooijman *et al*. (98) have described a differential expression of type 1 IGF receptors in relation to the stage of activation and differentiation of human T lymphocytes. Interestingly, in *Igf2* transgenic mice, the increased thymic cellularity is associated with a stimulated generation of phenotypically normal T cells, in particular CD4 T cells (99). In our hands, using radiobinding assays, specific type 2 IGF binding sites were detected on a murine immature T cell line (RL12−NP), as well as on Jurkat T cells (100). Only IGF–2 (and to a lesser extent, IGF–1) but not insulin could compete with [^{125}I]-IGF-2, with an ED50 around 10 nM. By affinity cross-linking, the IGF binding site expressed by lymphocytes was found to have a molecular weight (± 260 kDa) similar to the type 2 IGF receptor described on other cells. Treatment of murine FTOC with a polyclonal Ab against type 2 IGF receptor inhibited the early steps of T cell differentiation, with an increase of CD4−8− (double negative) and a decrease of CD4+8+ (double positive) thymocytes (101).

Some principles and advantages of thymic T cell education to neuroendocrine self-antigens

A model has been proposed according which neuroendocrine-related thymic polypeptides engage two distinct types of interactions with pre-T cells depending on their involvement as *self-antigens* of their family or as *cryptocrine signals* (Table 2 (102)), The interaction of neuroendocrine self-antigens with their corresponding TCR implies a binding of moderate affinity (10^{-6}–10^{-8} M), but with a high selectivity. Neuroendocrine self-antigens usually correspond to peptide sequences of neuroendocrine precursors which have been highly conserved during evolution. On the other hand, cryptocrine signalling between thymic neuroendocrine-related peptides and their cognate receptors expressed by pre-T cells implies a high-affinity binding(10^{-10}–10^{-11} M), but with a low selectivity. Moreover, a hierarchy of dominance appears in the organization of the polypeptide repertoire expressed in the thymus (Table 1). This is significant since self-tolerance primarily concerns self-determinants that are dominant on self-molecules (103–105).

Some selective advantages appear from this model of thymic neuroendocrine-related precursors of cryptocrine signals and self-antigens in T cell positive and negative selection, respectively. A first advantage is the absence of a tight allelic restriction in thymic T cell education to neuroendocrine families. Such an allelic restriction of central T cell tolerance of neuroendocrine families was hardly conceivable and our data seem to indicate that it is not the case in reality. Concerning the presentation of thymic OT for example, our data suggest that, though MHC class I molecules are involved in the process, it is the invariant neurophysin domain of the hybrid membrane 55-kDa protein that may bind OT for presentation to pre-T cells. Another selective advantage resides in the potential presentation to pre-T cells of the structure characteristic of the neurohypophysial family. With regard to the thymic presentation of NT, there is no physical constraint for a non-covalent binding to MHC since this neuropeptide is a linear peptide (in contrast to cyclic OT and IGF-2). In addition, the C-terminal sequence of NT includes tyrosine, isoleucine and leucine residues which can all be used in the anchorage to most of the MHC class I alleles. Given these characteristics, it is logical to postulate that NT and NT-derived C-terminal fragments could behave as natural ligands for a majority (if not all) of MHC class I alleles. This hypothesis is also in agrement with the high degree of conservation of NT-related C-terminal region throughout evolution (106).

For IGFs, the role of binding and transport proteins is

TABLE 2. The Dual Role of Thymic Neuroendocrine Peptides in T Cell Selection.

Cryptocrine signalling	Presentation of neuroendocrine self-antigens
Physiology	Physiology
Accessory signal in T cell development and activation (*positive* selection)	T cell education to neuroendocrine families (*negative* selection of self-reactive T cells)
Pathophysiology	Pathophysiology
Oversecretion in the bloodstream (paraneoplastic syndrome)	Failure (or breakdown) of immunological tolerance to neuroendocrine families
Involvement in the biology of T cell lymphomas	Autoimmune endocrine diseases
Pharmacology	Pharmacology
Immunomodulation by neuropeptide agonists or antagonists	'Reprogramming' of the immunological tolerance to neuroendocrine families
	'Tolerogenic' vaccination for prevention of autoimmune endocrine diseases

The interaction between neuroendocrine signals and receptors according to the cryptocrine model of cell signalling is involved in T cell positive selection and development. Thymic neuroendocrine precursors are also the source of neuroendocrine self antigens which are presented by thymic MHC class I molecules. This presentation could induce the negative selection or of self-reactive T cells oriented against neuroendocrine families.

ensured by IGF-binding proteins (IGFBPs). IGF-binding proteins have co-evolved with IGFs, but are not part of IGF precursors, and are encoded by distinct genes. These proteins play a prominent role in regulating the bioavailability and distribution of IGFs (107, 108). Interestingly, some IGFBPs are in close relationship with cell plasma membranes (through binding to integrins or the extracellular matrix), but their relationship with MHC as well as their potential implication in thymic IGF presentation to immature T cells deserve to be further investigated.

The immunological recognition of neuroendocrine families

In parallel with the increasing complexity of cell-to-cell signalling (from the primitive steps of autocrine, adhesion, paracrine and cryptocrine signalling to the (neuro)endocrine and complex neuronal networks in the central nervous system), there exists a counterpart in the organization of the genetic support responsible for chemical communication between cells. New members in neuroendocrine families were identified that were not classical endocrine hormones but mainly served as local growth- and differentiation-promoting autocrine/paracrine factors. Genes coding for these growth factors are predominantly expressed during foetal life, and they play an important role in embryonic development and organogenesis. So, the neuroendocrine system, with all of its components, has evolved to organize the internal body (the 'self') with an increasing complexity in the cell-to-cell signalling pathways, the current highest level being observed in the neural nets of the human central nervous system (109). On the other side, the immune system has evolved to protect the identity of self from aggression by infectious non-self.

When approaching the problem of the immunological recognition of neuroendocrine protein families (Fig. 1), two levels should be distinguished. At the central level of the thymus, T cell recognition of neuroendocrine-derived self-antigens primarily induces deletion of self-reactive T cell clones by apoptosis or developmental arrest. As discussed before, T cell tolerance firstly concerns the dominant and highly conserved sequence peptides of proteins. Thus, even if

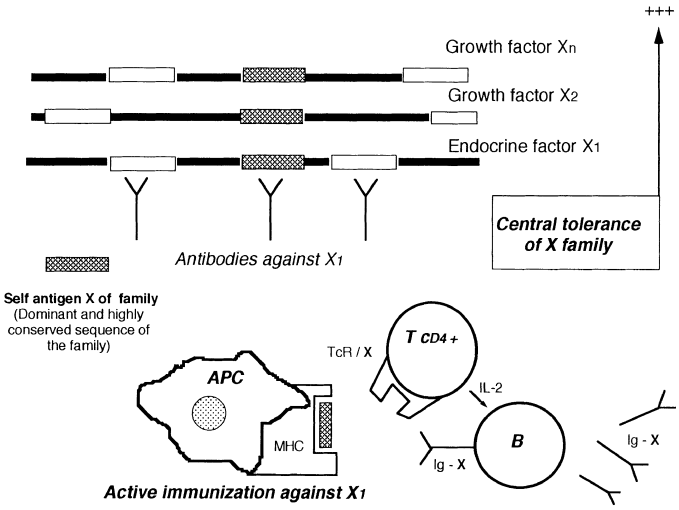

FIG. 1. The immunological recognition of neuroendocrine families. A neuroendocrine family X includes endocrine factors (X_1), as well as related tissue growth factors (X_2, X_n). Through its related thymic polypeptide, the central tolerance of a neuroendocrine family firstly concerns the dominant and highly conserved sequences of the family (self-antigen X). Because of the homology between the members of one family, the central tolerance is extended to all members, including the endocrine factors which are not or poorly expressed in the thymus. Active immunization against the hormone X_1 implies that some antigenic sequences are processed to be presented by dedicated antigen-presenting cells (APC) at the periphery, including the self-antigen X which is highly tolerated and against which specific immunoglobulins (Ig) will be hardly raised after a long immunization procedure.

some endocrine members of a family (i.e. VP or insulin) may be detected at low levels within the thymic microenvironment, the thymic tolerogenic function predominantly concerns their homologous dominant thymic growth factors (i.e. OT or IGF-2, respectively). Through the central tolerance of the dominant thymic factor however, the tolerogenic influence could extend to all members of the family.

At the peripheral level, active immunization againt a hormone X corresponds to the experimental breakdown of the immunological tolerance established for the family. For

those who have tried to raise Abs against an endocrine factor or against its homologous related tissue growth factors, it is a classic experience that the titre and the frequences of Abs against the endocrine factor are higher than those of Abs against related growth factors. Capra noted in 1975 that, early in immunization with neurophysins, the antisera did not cross-react with neurophysins of other species (110). Later, in a prolonged immunization procedure, extensively cross-reacting antisera were obtained. It is plausible that early antisera are directed against 'cryptic', and specific determinants of the molecule with low levels of tolerance; while later, when the tolerance of the whole family has been broken, the antisera are orientated against dominant, and highly conserved epitopes with high levels of tolerance.

A role played by a trouble of thymic T cell education in autoimmunity?

The development of an autoimmune disease affecting the neuroendocrine system may be viewed as a failure to develop or maintain tolerance to cellular or molecular components which are constitutively expressed by neuroendocrine cells (i.e. autoantigens such as insulin or GAD). Though the relationship between lymphoepithelial structures and autoimmunity has been suspected in 1962 by Burnet and Mackay (111), the question of a defective thymic T cell negative selection or self-education in the pathophysiology of autoimmune diseases has not been intensively investigated. Nevertheless, it has been shown that neonatal thymectomy prevents the emergence of diabetes in an animal model of autoimmune type 1 diabetes, the Bio-Breeding (BB) rat (112). In clinical practice also, thymectomy usually induces an improvement in patients suffering from autoimmune myasthenia gravis, especially when a thymoma (hyperplasia of thymic epithelium) is associated (113). In both cases, the benefit of thymectomy may result from the removal of the defective thymic censorship which is responsible for a continuous release and enrichment of the peripheral T cell pool with intolerant and self-reactive lymphocytes. The development of diabetes is prevented by the transplantation of thymus from diabetes-resistant (DR) to diabetes-prone (DP) BB rats (114). The transplantation of the thymus from non-obese diabetic (NOD) mice to DR mouse strains was also shown to induce diabetes in the recipients (115). While bone marrow transplantation is rather ineffective in preventing autoimmune diseases of MRL/+ mice, thymus transplantation is a crucial factor for their prevention (116). A defective process of thymic T cell negative selection has been suggested on the basis that the thymus of DR BB rats contains thymocytes predisposed to autoreactivity (117). Another argument is the observation that grafts of pure thymic epithelium from NOD mouse embryos to newborn C57BL/6 athymic mice induced CD4 and CD8 T cell mediated insulitis and sialitis (118). At the histological level, a defect in thymic function could be linked to a disorganization of the microenvironment, such as the giant perivascular spaces observed in the NOD mouse thymus (119), and the epithelial defects of BB rat thymus (120). Recently, we examined the elution profiles of ir-IGFs in the thymus from Wistar Furth (WF) normal rats, DR and DP BB rats. A peak of ir-IGF-2 >10 ng/mL was observed in the G75 profile of WF thymus extracts; a peak around 1.5 ng/mL was eluted from DR BB rat thymic extracts, while IGF–2 concentrations were almost undetectable in DP BB rats (121). Together, these observations strongly support the hypothesis that a defective thymic censorship or T cell self-education is actively involved in the pathophysiology of autoinimune type 1 diabetes.

Neuroendocrine self-antigens *versus* autoantigens: toward the design of tolerogenic vaccines for the prevention of autoimmune diseases?

Three types of factors are usually thought to be implicated in the pathogeny of autoimmune diseases:

1. The effector immune components are CD4 and CD8 autoreactive T cells which are specifically orientated against a given target cell or molecule. These autoreactive T cells result from a spontaneous breakdown of T cell tolerance, either at the central thymic and/or the peripheral level.
2. A series of extra- and intra-MHC genes are related to different autoimmune diseases. Some of these genes could intervene in the presentation of target autoantigens to autoreactive T lymphocytes, but others certainly not.
3. Finally, an environmental factor is involved and could be implicated in establishing a link between the target autoantigens and autoreactive T cells. A molecular mimicry between target autoantigens and micro-organisms may intervene at this level (122). The involvement of microbial superantigens has also been proposed to activate peripheral autoreactive T cells (123). A preventive strategy of autoimmune diseases can hardly be designed on the basis of the genetic components of autoimmune disease or the hazardous environmental factors. Manipulation of autoreactive T cells seems to be a more promising way by which an efficient prevention of autoimmunity can be envisioned.

In the neurohypophysial family, evidence has been presented that OT seems to be the neurohypophysial self-antigen. A strong immunological tolerance protects the OT lineage, more than the VP one, from autoimmune aggression. Indeed, some cases of idiopathic diabetes insipidus result from an autoimmune hypothalamitis orientated toward VP-producing neurones (124, 125). Given the implication of the OT lineage in the reproductive process, a stronger tolerance of this lineage is important for the preservation of the species. Thus, in the neurohypophysial family, while OT behaves as the self-antigen, VP is suspected to be one target autoantigen of the autoimmune process. As discussed previously, this conclusion is also supported by the frequences and the titres of Abs induced by active immunization against neurohypophysial peptides ($VP>>OT$). An infiltration of the hypothalamo-neurohypophysial tract by inflammatory mononuclear cells has been observed repeatedly, both after active immunization against VP (126), and in spontaneous diabetes insipidus (120). These observations suggest that hypothalamic magnocellular neurones express, on their surface, antigenic markers specific of their neurosecretory activity.

There is now significant evidence that insulin is one important autoantigen which is dealt with by various autoreactive

components of the immune system both in animal and human type 1 diabetes (127, 128). Moreover insulin is the specific marker of the pancreatic islet endocrine β cells. Oral, intranasal and parenteral administration of insulin or insulin-derived dominant autoantigens have been shown to inhibit the occurrence of diabetes in animal models of type 1 diabetes (129, 130). However, one cannot exclude the risk of priming or triggering autoinimunity by peripheral administration of an autoantigen (131). Reprogramming the immunological tolerance that is broken in autoimmunity is an attractive strategy for the prevention of devastating autoinimune diseases such as multiple sclerosis, rheumatoid arthritis or type 1 diabetes. Such reprogramming could be based upon the tolerogenic properties of the thymic epithelium. Instead of classical vaccination (with immune activation and induction of memory/immunocompetent cells), a form of tolerogenic vaccination is proposed that would lead to the deletion or the anergy of autoreactive T lymphocytes. The induction of T cell tolerance following peptide vaccination has already been obtained with synthetic peptides representing cytotoxic CD8 epitopes of T cells orientated against tumour antigens or viruses (132).

In the same perspective, the immunological barrier to xenogeneic grafts could be eliminated by transplanting at the same time the thymus of the donor, which could educate the immune system of the host to the self of the donor. In this case, it seems likely that tolerance to the xenogeneic graft would be enhanced if a competition between two self-educating schools is prevented by previous thymectomy of the recipient. This strategy has provided significant results in a pig-to-mouse model (133), and is currently tested for the transplantation of a pig 'thymo-kidney' to baboons (134).

Conclusion

A model is presented which recapitulates at the peptide level the dual role of the thymus in T cell negative and positive selection. This model is based upon the homology of peptide sequences between endocrine signals and related polypeptides that are synthesized in the thymic microenvironment and presented to differentiating T cells. This homology supports a dual role for the thymic repertoire of neuroendocrine precursors. First, they constitute a source of tolerogenic self-antigens; these self-antigens are highly conserved throughout evolution of their family. On the other hand, thymic neuroendocrine precursors deliver cryptocrine signals that provide accessory pathways in the process of T cell positive selection following their binding to neuroendocrine-type receptors expressed by developing T cells.

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