Central *Self*-Tolerance by Thymic Presentation of *Self*-Antigens and Autoimmunity

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Abstract: Before reacting against non-*self* infectious agents, the immune system is educated to tolerate the host molecular structure *(self)*. The induction of *self*-tolerance is a multistep process that begins in the thymus during fetal ontogeny (central tolerance) and also involves inactivating mechanisms outside the thymus (peripheral tolerance). The thymus is the primary lymphoid organ implicated in the development of competent and *self*-tolerant T cells. During ontogeny, T cell progenitors originating from hemopoietic tissues (yolk sac, fetal liver, and then bone marrow) enter the thymus and undergo a program of proliferation, T cell receptor (TCR) gene rearrangement, maturation and selection. Close interactions between thymocytes (pre-T cells) and the thymic cellular environment are crucial both for T cell development and induction of central *self*-tolerance. Thymic epithelial and stromal cells synthesize polypeptides belonging to various neuroendocrine families. The thymic repertoire of neuroendocrine-related precursors transposes at the molecular level the dual role of the thymus in T cell negative and positive selection. Thymic precursors not only constitute a source of growth peptides for cryptocrine signaling between thymic stromal cells and pre-T cells, but are also processed in a way that leads to the presentation of *self*-antigens by thymic major histocompatibility complex (MHC) proteins. Thymic neuroendocrine *self*-antigens often correspond to peptide sequences highly conserved during the evolution of their corresponding family. The thymic presentation of some neuroendocrine *self*-antigens is not restricted by MHC alleles. Following the presentation of neuroendocrine *self*-antigens by thymic MHC proteins, the T cell system might be educated to tolerate main hormone families. Recent experiments argue that a defect in the thymic essential tolerogenic function is implicated as an important factor in the pathophysiology of many autoimmune diseases.

INTRODUCTION

The whole field of immunophysiology is characterized by two fundamental properties. First, the generation of diversity of the immune system results from the random recombination in somatic cells of gene segments coding for the variable part of immunoglobulin (Ig) [1] B and T cell receptors for the antigen (TCR) [2]. Secondly, even before being able to recognize and react against non-*self* antigens, the immune system is educated to tolerate *self*-antigens. With memory and specificity, diversity and *self*-tolerance constitute the corner stones of immunophysiology. The induction of *self*-tolerance is not an automatic genetically programmed process, but is an acquired multi-step phenomenon that is initiated within the thymus during T cell differentiation [3].

In the last thirty years, close interactions were demonstrated between the major systems of cell-to-cell signaling, the nervous, endocrine and immune systems. Intimate neuroendocrine-immune interactions play a pivotal role in homeostasis as well as in normal development of different species. During phylogeny and ontogeny, the molecular foundations of the signaling systems emerge before the generation of diversity and

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specificity in the system of immune defenses. The objective of this review is to show how such intimate relationships between the neuroendocrine and immune systems already take place within the thymus, the primary organ involved in T cell lymphopoiesis. A special emphasis will be developed about neuroendocrine influences upon early T cell differentiation, as well as about the cellular and molecular mechanisms by which the T cell system is educated to tolerate neuroendocrine protein families. Recent observations will be reported that argue for an impairment of thymic T cell tolerance as an important component for the pathogenesis of autoimmune endocrine disorders such as Type 1 diabetes and Type 3 thyroiditis (Graves' disease). Finally, a rationale hypothesis is presented that supports the design of a tolerogenic approach based on thymic *self*-antigens for the prevention of autoimmunity.

ONTOGENY AND HISTOPHYSIOLOGY OF THE THYMUS

The thymus is a median organ in the superior mediastinum behind the sternum, and its general shape resembles the leaves of the 'thyme' plant. The major part of the thymic parenchyme is constituted by epithelial cells (TEC) which derive from (a) endoderm of the third pharyngeal pouch, and (b) ectoderm of the corresponding branchial clefts [4, 5]. Inclusion of ectoderm explains the heterogeneity of TEC, as well as the similar phenotype of medullar and outer cortical TEC. Interactions between the epithelial rudiment and cells derived from the cephalic neural crest cells are necessary for the development of a functional thymic structure [6, 7]. Some human diseases and related animal models are characterized by a defective thymic development that leads to primary immune deficiencies [8]. The DiGeorge's syndrome includes the congenital absence (or hypoplasia) of thymus, parathyroids, and defects in the heart and truncal vessels [9]. This syndrome seems to result from the failure of migration of the cephalic neural crest [10]. Mice in whom the homeobox *Hoxa-3* has been disrupted present thymic aplasia, parathyroid hypoplasia, and frequent defects in the heart and great vessels [11]. Wild animals with immunodeficiencies most closely resembling those of DiGeorge's syndrome are 'nude' mice with hairlessness and lack of thymic development both resulting from defect in epithelial cells. The nude phenotype is caused by mutations in a gene on murine chromosome 11 that encodes the transcription factor winged-helix nude (whn). In the absence of *whn*, the thymus rudiment still develops but is filled with primitive epithelial cells that do not specialize and segregate into subregions [12].

Thymic nurse cells (TNC) are large epithelial cells found in the subcapsular and outer cortex of the thymus in different species. TNC contain a large number of internalized thymocytes (immature or pre-T cells) that are not phagocytosed, but are engulfed by emperipolesis within caveoles delineated by TNC plasma membrane [13]. TNC-associated thymocytes display a high mitotic index. Functionally, TNC are involved in T cell selection since a TNC-derived cell line is able to induce *in vitro* deletion of thymocytes bearing a transgenic TCR [14]. Ultrastructural analyses have shown that TNC possess the intracellular machinery necessary for antigen processing and presentation [15].

The thymic stroma also contains non-lymphoid bone marrow-derived cellular elements: macrophages and dendritic/interdigitating (IDC) cells. Macrophages are dispersed throughout thymic cortical and medullary parenchyme, while IDC are located at the cortico-medullary junction. The expression by macrophages and IDC of major histocompatibility complex (MHC) class I and II molecules is linked to their activity as dedicated antigen-presenting cells (APC).

Thymic lymphoid cells (thymocytes) form a 'passenger' cell population of the thymus. First from yolk sac and fetal liver, then from bone marrow, T cell progenitors are attracted and migrate within the thymus. This organ provides an appropriate and specific microenvironment for T cell maturation. In the thymic network, several types of interactions between thymocytes and parenchymatous cells trigger T cell proliferation, TCR gene random rearrangements, differentiation end expression of the first specific CD T cell

markers, CD2 in humans and Thy-1 in mice. The pathways of T cell differentiation may be followed by the differential expression of the adhesion molecule CD44 (Pgp-1) and the α chain of the IL-2 receptor (CD25), the CD3/TCR complex proteins, and the co-receptor proteins, CD4 and CD8. Most steps of T cell differentiation occur in the cortex, while thymic medulla contains mainly mature T cells. From 100 T cell progenitors which migrate into the thymus, about only 5 to 10 mature T cells will leave it in a state of functional competence and *self*-tolerance [16]. Thus, the thymus is primarily a graveyard for T cells harboring a randomly rearranged TCR oriented against *self*-antigens encountered and presented in the thymus.

THE THYMIC REPERTOIRE OF NEUROENDOCRINE *SELF*-ANTIGEN PRE-CURSORS

1. Thymic Neurohypophysial Self-Antigen [See Complete Review in 17]

Though the galactogogue action of thymic extracts had already been reported at the beginning of this century [18], oxytocin (OT) was identified as the primary mediator of galactokinesis only in the early 50's [19]. OT and vasopressin (VP) are nonapeptides that are synthesized by distinct neurons of the hypothalamic paraventricular and supraoptic nuclei. Hypothalamic *OT* and *VP* transcription is followed by mRNA translation into larger precursors that are processed during their axonal transport toward the neurohypophysis. This processing gives rise to the active neurohormones OT and VP, and their associated 10-kDa binding proteins neurophysins. From the neurohypophysis, OT and VP are released with neurophysins in the bloodstream. According to the neuroendocrine type of cell-cell signaling, they are transported in the bloodstream to their target receptors in the mammary myoepithelial gland and myometrium for OT, and in the kidney collecting tubules and vessel smooth muscle cells for VP.

The oxytocic activity of thymic extracts was not further characterized until 1986 when immunoreactive (IR) OT and neurophysin were identified in the human thymus [20]. The other neurohypophysial hormone vasopressin (VP) is also detected in the human thymus but IR VP concentrations are much lower (0.01-0.06 ng/g VP *vs*. 2.2-18.4 ng/g OT). By immunocytochemistry (ICC) and by *in situ* hybridization, TEC/TNC from different species were shown to express neurohypophysial genes, and the use of specific monoclonal antibodies (mAbs) against OT and VP revealed a dominance of OT immunoreactivity. However, *OT* expression and OT synthesis in TEC/TNC is not associated with the secretion of the nonapeptide or neurophysin in the supernatant of human TEC primary cultures. As another argument against a classic neurosecretion of thymic OT, the peptide is not located in secretory granules but is diffuse in the cytosol, in vesicles of the endoplasmic reticulum, or associated with cytokeratin filaments [21]. Similar ultrastructural features were also described for *OT*- and *VP*-expressing murine splenic eosinophils [22].

The hypothesis that thymic OT behaves as the *self*-antigen of the neurohypophysial family was investigated through different types of experiments. Using affinity chromatography with a mAb to the monomorphic part of MHC class I proteins, a 55-kDa protein was identified in a preparation of human TEC plasma membranes. This protein was stained both with mAb to MHC class I and with a polyclonal Ab to neurophysin. This protein is thought to be a hybrid protein with a neurophysin domain (10 kDa), and a MHC class I heavy chain domain (45 kDa) [23]. A 40-kDa protein isolated from small-cell lung cancer with anti-neurophysin Abs also reacted with a mouse mAb to MHC class I. Edman degradation on this 40-kDa protein revealed a N-terminal sequence from MHC-class I protein [24]. According to this interpretation of a hybrid neurophysin/MHC class I protein, the processing of thymic OT would implicate MHC pathways for targeting to the TEC plasma membrane. By analogy with the situation in the hypothalamo-neurohypophysial axis, the neurophysin domain of the thymic OT precursor could bind and transport OT until the external limits of OT-synthesizing TEC. If this hypothesis were confirmed, two significant advantages would appear in thymic T cell tolerance of the

neurohypophysial family: (1) the absence of a MHC allelic restriction such as in the peripheral antigen presentation by professional APC [24], and (2) the presentation to immature T cells of the classic structure of neurohypophysial peptides (a cycle of six amino acids closed by a disulfide bridge, and a linear C-terminal part of three residues). The antigenic behavior of thymic OT was confirmed by the fact that the immunological recognition of membrane OT by specific mAbs markedly enhances the secretion of interleukin-6 (IL-6) and leukemia inhibitory factor (LIF) in primary cultures of human TEC [26]. The treatment of such cultures with a mAb to VP did not induce any significant effect on IL-6 and LIF secretion arguing for the absence of VP presentation by thymic TEC. Additional observations about the tolerogenic function of thymic OT will be presented below.

2. Thymic Insulin-Related Genes

The presence of a thymic insulin-like peptide was first reported in 1965 [27] on the basis that AKR female mice spontaneously develop hypoglycemia and thymic hyperplasia associated with lymphoid leukemia. Marked hypoglycemia was induced by injection of mouse thymic extracts into female AKR mice, and this biological activity exceeded the hypoglycemic potency of similarly prepared pancreatic extracts. To the best of our knowledge, this thymic insulin-like peptide was not further characterized until independent observations showing that insulin-like growth factor (IGF) [28] and insulin genes [29] are expressed in the thymus. Using a panel of Abs directed against distinct insulin-related polypeptides, ICC analyses revealed that IGF-2 is the dominant member of the insulin family expressed by TEC/TNC in different species [30]. The components of the IGF axis, including IGF-binding proteins (IGFBPs), have been characterized in the human thymus. Human TEC express different members of the IGF axis, with a predominance of IGF-2 and IGFBP-2 to –6 [30]. RT-PCR with specific primers showed that thymic *IGF2* transcription is controlled by the same promoters as in other fetal and adult extrahepatic tissues [31]. IR (pro)insulin was not detected in the thymic parenchyme, whereas IR IGF-1 was detected in thymic stromal cells with a macrophage-like morphology and distribution. The expression of *IGF* and *IGF receptor* (*IGFR*) genes was investigated by RT-PCR during ontogeny of the murine thymus. *IGF1*, *IGF1R*, *M6P/IGF2R* are expressed in the thymus both in fetal and postnatal life, whereas *IGF2* transcripts decline after birth but remain detectable on the seventh week. By *in situ* hybridization, *IGF2* mRNAs were located in the outer cortex and medulla of the postnatal thymus, in accordance with the distribution of IR IGF-2 [32]. In the human thymus, IGF-2, IGF-1 and (pro)insulin concentrations are: 96.7 ± 10.6 ng/g, 42.9 ± 5.0 ng/g, and < 0.01 ng/g wet weight, respectively. No secretion of IGF-2 or IGF-1 could be evidenced in primary cultures of human TEC. By ICC and confocal microscopy, a significant part of IR IGF-2 (but neither IR IGF-1, nor IR proinsulin) was detected at the outer surface of human TEC plasma membrane. A thymic hyperplasia is observed in transgenic mice overexpressing *IGF2* under the control of the MHC H-2K^b promoter [33]. Altogether, these observations argue that the thymic insulin-like factor described by Pansky and coworkers corresponds to IGF-2. The close homology between IGF-2 and proinsulin may explain cross-reactivity with polyclonal Abs to insulin that were used in 1965. Similarly, the hypoglycemic effects of thymic extracts may result from the binding of thymic IGF-2 to insulin receptors. Moreover, the syndrome of hypoglycemia and lymphoid leukemia associated with thymic hyperplasia in AKR female mice might result from over-expression of *IGF2* in hyperplastic thymic epithelium leading to IGF-2 secretion into the bloodstream and disturbed thymic T cell lymphopoiesis.

3. Other Components of the Thymic Repertoire of Neuroendocrine-Related Pre-cursors

Neurokinin A (NKA) is the peptide of the tachykinin family expressed in human and rat TEC under the control of the preprotachykinin A gene (*PPT-A*) [34]. NKA is known to exert IL-1-like mitogenic effects on murine thymocytes [34]. The

ß and ɣ forms of *PPT-A* mRNA also encodes substance P (SP) in the brain, but this tachykinin is not detected in thymic epithelium suggesting a differential processing or translation of *PPT-A* mRNA in neurons and TEC. Interestingly, while IR NKA has been identified in TEC, IR SP was detected only in nerve profiles associated with thymic blood vessels. Since high affinity SP receptors have been described in association with vascular structures of rat thymic medulla [36], it is likely that neuronal SP regulates local blood flow.

IR neurotensin (NT) and somatostatin have been identified in sparse stromal cells of the chicken thymus [37]. Primary cultures of human TEC contain ± 5 ng/g IR NT/10^6 cells, of which 2.5-5% is associated with TEC plasma membranes. However, IR NT was not detected in the culture medium further questioning the classic neurosecretory model for thymic epithelium. Using anti-MHC class I affinity chromatography followed by HPLC analysis, one peak of IR NT was eluted at the same position as synthetic NT_{1-13}, together with two other NT C-terminal smaller fragments [38].

THYMIC NEUROENDOCRINE PEPTIDES AND T CELL DEVELOPMENT

1. Thymic OT and Focal Adhesion

The active role of thymic OT in a cryptocrine type of signaling between TEC and thymocytes was first evidenced by the expression of specific neurohypophysial binding sites by murine pre-T RL12-NP and cytotoxic CTL-L_2 cell lines. These binding sites behave as functional receptors since they transduced neurohypophysial signals through the phosphoinositide pathway [39]. The molecular identity of these neurohypophysial receptors remains to be further precised (OT or a V1-subtype), and this point is under current investigation. Nevertheless, the KD of these receptors concords with the concentration of IR OT quantified in the human thymus, and mitogenic properties of neurohypophysial peptides are associated with the increase of inositol phosphates in pre-T cells [39]. A very recent study has underlined the importance of phosphoinositide 3-kinases in T cell development and activation, as well as neutrophil migration (but without any significant role in the development and function of B cells) [40].

The observation of numerous points of focal adhesion between OT-producing TEC and pre-T cells [21] prompted us to investigate the potential implication of the recently discovered focal adhesion-related kinase $p125^{FAK}$ [41]. Protein tyrosine phosphorylation is known to be an early event in T cell activation. Western analysis of RL12-NP proteins probed with with anti-phosphotyrosine (PY-20) revealed a number of proteins the phosphorylation of which increased after OT or VP treatment. OT-mediated phosphorylation was rapid and reached a maximum within 1-5 min. OT also was more potent than VP to induce phosphorylation in RL12-NP cells. Two of these proteins were precipitated with anti-FAK mAb 2A7 and were identified one as $p125^{FAK}$ and the other as a coprecipitated 130-kDa protein (most probably $p130^{Cas}$) [42]. A neurohypophysial V1 antagonist inhibited OT-induced phosphorylation of $p125^{FAK}$, which demonstrates the specificity of this action but also questions the identity of the natural neurohypophysial ligand for V1 receptors expressed by T lymphocytes. Another protein phosphorylated by OT in pre-T cells was identified as paxillin, a 68-kDa protein located at focal adhesion sites in association with $p125^{FAK}$ [42]. Since T cell differentiation depends on close interactions and adhesion between thymic stromal cells and thymocytes [43], the implication of focal adhesion kinases in this process surely deserves to be further investigated. Altogether, these observations largely document the model of cryptocrine signaling proposed to distinguish the chemical communication between TEC/TNC and immature T cells that migrate and differentiate at their contact [44].

2. Thymic IGFs and T Cell Differentiation

Murine fetal thymic organ cultures (FTOCs) are an appropriate *in vitro* model for the study and manipulation of T cell differentiation [45-47]. The thymus removed from murine embryos on the 14^{th} fetal day contains the epithelial rudiment and only

immature T cell progenitors. After seven days in culture, pre-T cells differentiate along usual pathways. Thus, FTOCs closely mimic physiological conditions of *in vivo* T cell differentiation. Briefly, the phenotype of early T cell progenitors is double negative for the expression of CD4 and CD8 (CD4-CD8-). Then, they become double positive (CD4+CD8+), acquire CD3, and finally turn into the single positive cells expressing either CD4 or CD8. With the characterization of the thymic IGF axis, several observations have been reported supporting the implication of IGFs in T cell development. Thymocytes express both types of IGF receptors (IGF-1R and M6P/IGF-2R) [48-50]. Administration of IGF-1 stimulates stimulates lymphopoiesis and modulates the regeneration of T lymphocytes in a rat model of dexamethasone-induced apoptosis [51, 52]. The thymus of *IGF2* transgenic mice displays an increase thymic cellularity, with a higher number of the CD4+ T cell subset [53]. FTOC treatment with an anti-IGF-2 mAB, an anti-IGF-1R, or an anti-M6P-IGF-2R polyclonal Ab induced a blockade of T cell differentiation at the CD4-CD8- stage. This was evidenced by an increase of CD4-CD8- cells and a parallel decrease in the percentage of CD4+CD8+ thymocytes. Anti-IGF-2 Ab also induced an increase in CD8+ cells suggesting that thymic IGF-2 might have a role in determining differentiation into the CD4 or CD8 lineage. The strongest effects upon T cell proliferation and differentiation were observed in FTOCs treated with anti-IGF-1R mAb. Anti-IGF-1 Ab decreased the percentage of CD4-CD8- cells and increased the frequency in CD4+CD8+. Though the proinsulin gene is transcribed in the murine thymus [25, 54], FTOC treatment with anti-(pro)insulin did not exert any significant effect on T cell differentiation. As shown by these data, the intrathymic IGF-mediated signaling plays an active role in T cell differentiation during ontogeny [32].

THE NATURE OF *SELF*: THYMIC NEUROENDOCRINE *SELF*-ANTIGENS AND *SELF*-TOLERANCE

During their thymic differentiation, immature T cells randomly rearrange the gene segments coding for the variable part of their TCR. Many of these TCR recombinations are oriented against *self*-antigens expressed and presented by MHC proteins in the thymic microenvironment. The interaction of *self*-reactive T cells with their cognate *self*-antigens is thought to lead to their negative selection either by programmed cell death (apoptosis) or by developmental arrest. Thymic clonal deletion was demonstrated using mouse mammary tumor virus (MMTV)-encoded superantigens [55], and with transgenic mice expressing a TCR specific for the male antigen H-Y [56]. *Self*-antigens 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 [57]. The 'avidity-affinity' hypothesis has been proposed to explain this major paradox of thymic physiology, i.e. how *self*-antigens are able to condition both the death and the survival of T cells ? According to this hypothesis, T lymphocytes are deleted if their TCR is strongly engaged with a *self*-antigen at high concentrations (10^{-6} M). On the contrary, they are positively selected if their TCR is barely engaged with *self*-peptide at low concentrations (10^{-12} M) [58, 59]. However, the affinity of a TCR for its cognate antigen is rather low (10^{-8} M at maximum). Thus, it is now of crucial importance to know the nature and the amount of peptide/MHC combinations that contribute *in vivo* to T cell negative and positive selection [60].

As another explanation to this paradox, it has been proposed that the thymic repertoire of neuroendocrine-related precursors recapitulates at the molecular level the dual role of the thymus in T cell selection [61]. Thymic polypeptide precursors engage two distinct types of interactions with pre-T cells depending on their behaviour either as cryptocrine growth signals or as *self*-antigens representative of their family. Cryptocrine signaling implies a high-affinity (10^{-12} M) but poorly specific binding of thymic neuroendocrine peptides to their cognate receptors expressed by pre-T cells. Such cryptocrine signaling has been shown to exist and to be mediated by OT and IGF-2 in the thymus network. Recently, other authors have also discussed the point that thymocyte selection cannot be explained

by interaction with TCR alone [62]. On the other hand, neuroendocrine *self*-antigens bind to their corresponding TCR with a moderate affinity (10^{-8} M), but with a high specificity. This latter interaction is thought to induce the central T cell tolerance of neuroendocrine families. Neuroendocrine *self*-antigens usually correspond to peptide sequences of neuroendocrine precursors highly conserved throughout evolution of a given hormone family. Moreover, a hierarchy of dominance has been shown in the organisation of the neuroendocrine polypeptide repertoire expressed in the thymus. This hierarchy is highly significant since *self*-tolerance primarily concerns *self*-antigenic determinants that are dominant on *self*-proteins [63, 64]. Thus, even if some members of a family (i.e. VP or proinsulin) are detected at very low levels in the thymus, the thymic tolerogenic function firstly concerns their homologous dominant thymic growth factors (*i.e.* OT or IGF-2, respectively). Through the central tolerance of the dominant factor however, the immune system could be educated to tolerate the whole family.

The tolerogenic properties of OT and IGF-2 have not been definitively proved. However, OT-mediated functions are known to be stronger tolerated than the VP-mediated ones. Some cases of 'idiopathic' central diabetes insipidus result from an autoimmune hypothalamitis directed against VP-producing neurones [65, 66]. Given the implication of OT as a reproductive hormone, a stronger tolerance of the OT lineage is crucial for the preservation of the species. This conclusion is indirectly supported by the frequence and the titers of Abs obtained from active immunization (equivalent to tolerance breakdown) against neurohypophysial hormones (VP >> OT). Similar conclusions were drawn from active immunization against insulin, IGF-1 and IGF-2 (see after). Thus, in the neurohypophysial peptide family, while OT behaves as the ***self***-antigen, VP is strongly suspected to be the **auto**antigen targeted by the autoimmune process leading to some forms of central diabetes insipidus. In the insulin hormone family, insulin is a major autoantigen of the autoimmune process against insulin-secreting islet ß cells, and insulin immunogenicity might result from its very low expression in the thymus network.

Using RT-PCR, *in situ* hybridization and ICC, we recently investigated the ontogeny of neurohypophysial gene expression in the thymus of Balb/c mice. Transcripts of *OT* and *VP* were detected without any visible modulation in the thymus already from fetal day (FD) 14 until day 7 after birth [67]. In the murine thymus, neurohypophysial trancripts are located in cells with an epithelial morphology and are absent in the lymphoid compartment. Because of the microscopic size of thymic rudiments before FD 14, it was not possible to analyze the earlier thymic expression of neurohypophysial genes. Nevertheless, the comparison with previous reports shows that the transcription of neurohypophysial genes in the rodent thymus precedes their expression in the magnocellular neurones of the hypothalamic-neurohypophysial axis. At the peptide level, this difference is more evident since IR OT is detected in the thymus on FD 15, whereas ICC labels IR OT in the hypothalamus only on FD 20. Thus, the expression of neurohypophysial genes in the murine thymus coincides with the appearance of T-cell progenitors and precedes their hypothalamic transcription. This observation is significant with regard to the physiological role proposed for thymic OT as a *self*-antigen involved both in T cell lymphopoiesis and in central tolerance of the hypothalamo-neurohypophysial functions. Indeed, it is logical that the induction of central *self*-tolerance precedes the appearance of antigenic epitopes in target organs susceptible to autoimmune aggression.

THYMUS DYSFUNCTION AND AUTO-IMMUNITY

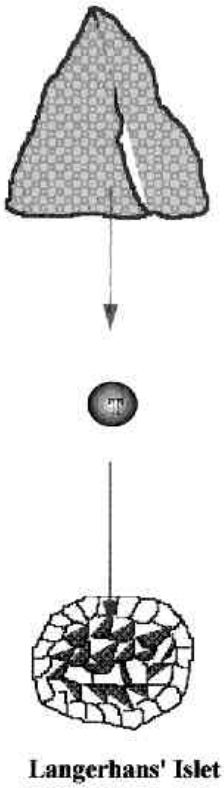
Though the relationship between lymphoepithelial structures and autoimmunity has been suspected already in 1962 by Burnet and Mackay [68], the question of a defective central T-cell *self*-tolerance in the pathophysiology of autoimmune diseases has not been intensively investigated. Also, Burnet proposed that the emergence of 'forbidden' *self*-reactive clones

plays a major role in the pathophysiology of autoimmunity. These 'forbidden' *self*-reactive clones were initially thought to result from somatic mutations during development. Since the whole immune repertoire is generated by somatic random recombination, this hypothesis is no longer tenable. However, some 'holes' in the peripheral T cell repertoire could appear if some *self*-reactive lymphocytes would not be deleted by their cognate *self*-antigen.

Neonatal thymectomy prevents the emergence of diabetes in an animal model of autoimmune Type 1 diabetes, the Bio-Breeding (BB) rat [69]. In clinical practice also, thymectomy induces a significant improvement of patients suffering from autoimmune myasthenia gravis [70]. In both cases, the benefit of thymectomy may be explained by the removal of the defective thymic censorship. At least theoretically, such a trouble in thymic *self*-tolerance would be responsible for a continuous release and enrichment of the peripheral T-cell pool with intolerant and 'forbidden' *self*-reactive lymphocytes. The transplantation of the defective thymus from non-obese diabetic (NOD) mice to athymic nude mice induces autoimmune insulitis in the recipient [71]. On the contrary, the development of autoimmune diabetes is prevented by the transplantation of thymus from diabetes-resistant to diabetes-prone BB rats [72]. Recently also, insulitis and sialitis developed in athymic nude mice grafted with pure thymic epithelium from NOD mice [73]. Some studies also suggest that thymic epithelium not only mediates negative selection of *self*-reactive T cells, but also selects regulatory T cells helping in maintaining tissue-specific *self*-tolerance [74].

1. Autoimmune Type 1 Diabetes (Fig. 1)

More and more, the breakdown of immunological tolerance of insulin-secreting islet ß cell is thought to be a major event in the pathophysiology of autoimmune Type 1 diabetes and several putative ß cell autoantigens have been identified [75]. In this cohort of autoantigens, insulin (and/or its precursor proinsulin) is specific



Thymus physiology

1. Negative selection of *self*-reactive T cells (90-95%)
2. Development of competent T cells (5-10%)

IGF-2 = dominant thymic peptide of the insulin hormone family

Thymus pathophysiology

Abnormal cytoarchitecture [76]
Genetic susceptibility
Absence of intrathymic transcription of **IGF-2** gene ***(IGF2)***
- Selection and enrichment of the peripheral T cell repertoire with 'forbidden' *self*-reactive T cells to insulin family
- Lymphopenia [76]

Role of environment (virus, gonadal steroids, thymic innervation) ?

Molecular bridge between *self*-reactive T cells and islet ß cells

- **Genetic susceptibility** (MHC +++)
- **Environmental factors:**
  - Molecular mimicry
  - Nutrition
  - Stress
  - ...

Fig. (1). Role of the thymus in the pathophysiology of autoimmune Type 1 diabetes.

of the islet ß-cell-related autoantigens and plays an important role in the development of this chronic devastating disease. However, despite the identification of ß-cell antigens, the origin of *self*-reactivity in Type 1 diabetes remains unexplained. Noteworthy, a defect of thymic epithelium was previously observed in BB rats and NOD mice [76, 77]. This epithelial defect was proposed to intervene in the dysfunction of thymic T cell differentiation and susceptibility to autoimmune diabetes. To investigate the hypothesis of a defect in thymic T cell *self*-tolerance of the insulin family in that disease, a comparative study of thymic *IGFs* and *insulin* (*INS*) gene expression was performed in BB rats. The absence of thymic *IGF2* expression was evidenced in more than 80% of diabetes-prone BB rats, while *IGF1* and *INS* mRNAs were detected in Wistar-Furth, diabetes-resistant and diabetes-prone BB rats. This defect was thymus-specific since diabetes-prone BB rat brains and livers express readily detectable *IGF2* mRNA. The defect was shown both at the *IGF2* transcript and IGF-2 protein levels. The absence of *IGF2* expression in the thymus of young and adult diabetes-prone BB rats might have a role in the defect of central *self*-tolerance of the insulin hormone family and contribute to the pathophysiology of autoimmune Type 1 diabetes [78; manuscript submitted]. Interestingly, the region containing the *IGF2* locus on chromosome 1 in spontaneously diabetic BB rats is a protective locus [79], but is also associated with blood glucose in diabetic rats [80].

A recent debate was opened about the relative contribution of thymic IGF-2 and insulin to *self*-tolerance of the insulin family. The *IDDM2* locus of susceptibility to diabetes maps to a variable number of tandem repeats (VNTR) mini-satellites on chromosome 11p15, upstream of *INS* and *IGF2* genes [81]. However, there exists some controversy about the precise boundaries of *IDDM2* [82]. Short class I VNTR alleles (26-63 repeats) predispose to Type 1 diabetes, whereas long class III alleles (140-210 repeats) are dominantly protective [83]. Two independent studies reported that higher levels of *INS* mRNA were associated with the protective class III VNTR alleles of the *IDDM2* locus [84, 85]. The susceptibility locus VNTR is also associated with *IGF2* expression in humans [86], though no significant influence of VNTR susceptibility (class I) or protective (class III) alleles could be evidenced on *IGF2* transcripts in the human fetal thymus [87]. Besides this association, the tolerogenic effect of thymic insulin has been deduced from its expression in dendritic cells that are professional antigen-presenting cells.

The pattern of immune reactivity generated by IGF-2 has not been investigated until now. With regard to the cellular site of *INS* and *IGF2* expression, both thymic dendritic and TEC are able to present antigens. Functionally, the dominance and crypticity of T-cell epitopes [88] determine the final orientation of an antigen-driven response, towards either priming or tolerizing. Thus, if a tolerogenic response indeed follows the intrathymic expression of an insulin-related protein, this firstly concerns the dominant peptide of this family. Thymic IR (pro)insulin concentrations (± 2 pmol/mg protein) are much lower than thymic IR IGF-2 (± 100 ng/g wet weight), and it is known that thymic tolerance rapidly decreases for peptide concentrations below 10 nM [58, 59]. As another question to the physiological significance of thymic insulin, FTOC treatment with a mAb to (pro)insulin does not influence T-cell differentiation [32]. The crucial role of IGFs in fetal and postnatal development implies that these growth factors must be strongly protected from an autoimmune attack. As an important though indirect argument for the tolerogenic properties of IGF-2, the production of specific Ab is much more difficult with IGF-2 than with IGF-1 or insulin [89]. IGF-2 protein contains peptide sequences highly conserved during evolution of the insulin family [90]. Through this homology, thymic IGF-2 would be a good candidate for inducing central immunological tolerance of the insulin family although the tolerance of insulin *per se* would be weaker. This might explain why B- and T-cell reactivity to insulin has been observed in diabetic and non-diabetic individuals [91]. Contrary to other autoantigens implicated in Type 1 diabetes (like GAD 65 and phosphatase IA-2), insulin is specifically synthesized and secreted by islet ß

cells, and insulin is considered as a critical target of the autoimmune diabetogenic process [92]. Insulin is also the only autoantigen whose gene maps to a diabetes susceptibility locus [83]. Again, insulin immunogenicity might result from its very low expression in the thymus network. Oral, intranasal and parenteral administration of insulin has been shown to inhibit the occurrence of diabetes in NOD mice [93], but the same treatment is ineffective [94], or even promotes disease in diabetes-prone BB rats [95]. Thus, current insulin-based preventive strategies cannot ignore the risk of priming autoimmune Type 1 diabetes as a result of autoantigen administration [94]. A crucial question also concerns both the existence and the putative diabetogenic role of 'forbidden' IGF-2 *self*-reactive T cell clones. This question will be examined using *IGF2*-deleted mice in whom IGF-2 is no more a *self*-antigen so that tolerance of IGF-2 should be very low. The problem is nevertheless quite complicated since *IGF2* is subject to tissue-specific parental imprinting in mice as in humans [97-99].

2. Autoimmune Type 3 Thyroiditis (Graves'-Disease)

The pathogenesis of autoimmune thyroid diseases involves several thyroid antigens including human sodium iodide symporter, thyrotropin receptor (TSH-R), thyroid peroxydase (TPO) and thyroglobulin (Tg) [100]. Auto-Abs to TSH-R exerting thyroid stimulation by recognition of TSH-R are responsible for the state of hyperthyroidism in autoimmune Type 3 thyroiditis or Graves'disease [101]. Thyroid-associated orbitopathy and pretibial myxedema are commonly associated in Graves'disease and *TSHR* and/or a *TSHR* variant [102] was shown to be expressed in orbital tissues [103] and in pretibial fibroblasts of patients with Graves'disease [104]. T cell recognition of TSH-R peptide sequence 158-176 is thought to be an early event in the initiation of the autoimmune process leading to Graves'disease [105]. During their thymic development, T cells can be educated to tolerate thyroid-related epitopes since a TSH-R variant [106], TSH-R itself, and other thyroid antigens [106-108] are expressed in the human thymus. The failure of thymic T cell tolerance of thyroid antigens has not been demonstrated, but this hypothesis is supported by the increased thymic size and density observed by computed tomography in patients with Graves'disease [107].

CONCLUSIONS AND PERSPECTIVES IN TOLEROGENIC VACCINATION

The thymic repertoire of neuroendocrine *self*-peptide precursors recapitulates at the molecular level the dual physiological role played by the thymus in T cell positive and negative selection. Thus, this model provides some answer to the thymic paradox in T cell development and deletion. During the last fifteen years, the advancement of our common knowledge in thymus physiology has been impressive. It is not unreasonable to consider that the natural tolerogenic properties of the thymus could be useful in organ transplantation (including xenotransplantation) the ultimate goal of which is complete tolerance between the donor and the recipient [109, 110]. In addition, more and more experimental observations suggest that a defect in the active establishment of thymic T cell *self*-tolerance is implicated as a crucial event in the pathogenesis of organ-specific autoimmune diseases. As already claimed by Lederberg in 1959 [111], the most efficient way to deal with autoimmunity is to delete it. Of course, it is neither useful nor even ethical to propose thymectomy for the prevention or treatment of chronic autoimmune diseases. Nevertheless, the identification of neuroendocrine *self*-antigens that are presented by thymic MHC proteins could be useful to provoke deletion or inactivation of 'forbidden' *self*-reactive T cell clones that have escaped the induction of thymus *self*-tolerance (Fig. **2**). While autoantigens are the drivers of autoimmune process, thymic *self*-antigens could be used to reprogram *self*-tolerance, and peptide vaccination is able to induce T cell tolerance [112, 113]. In addition to classic immunogenic vaccination, perhaps will we have soon at our hand tolerogenic vaccination for the prevention of autoimmunity, the heavy tribute mainly paid by the human species for the specificity and diversity of its immune system.

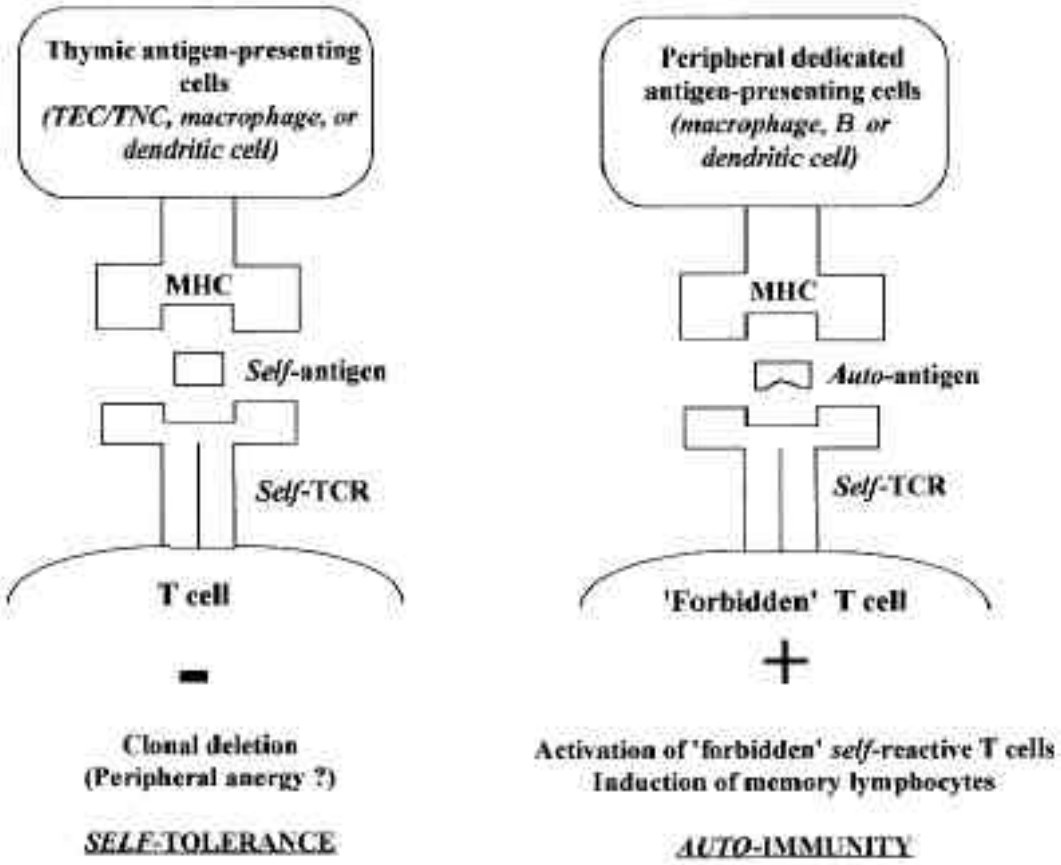


Fig. (2). Two opposite immune responses driven by two types of antigens:
T cell tolerance is elicited by a ***self***-antigen, whereas T cell activation follows presentation of an **auto**antigen.

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