MINI-REVIEW

Quantification of T cell receptor rearrangement excision circles to estimate thymic function: an important new tool for endocrine–immune physiology

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Abstract

Although the thymus constitutes a target organ for most protein and steroid hormones, it has been quite difficult to determine the precise control exerted *in vivo* by the endocrine system upon thymic function. The biological role of the thymus is to ensure the generation of a diversified population of peripheral T cells able to respond to non-self-antigens but nevertheless tolerant to self-antigens. For a long time, thymic function could not be monitored, as a consequence of the absence of adequate technology to differentiate recent thymic emigrants from naive T cells. The generation of T cell receptor (TCR) diversity occurs in the thymus through recombination of gene segments encoding the variable parts of the TCR α

and β chains. During these processes, by-products of the rearrangements are generated in the form of TCR excision circles (TRECs). As these molecules are lost upon further cell division, their quantification is actually considered as a very valuable tool to estimate thymic function. The most appropriate TREC is δ Rec- Ψ J α TREC or signal joint TREC resulting from δ Rec- Ψ J α rearrangement (TCRD deletion) that occurs late during thymopoiesis, before V α -J α rearrangement. Here we describe how TREC quantification is a powerful and reliable method to evaluate the impact of hormones and endocrine disorders upon thymic function.

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The place of the thymus in the endocrine system

During embryonic development, the thymic and inferior parathyroid epithelial rudiments both originate from the endoderm of the third pharyngeal pouch. The thymic epithelial anlage is then colonised by cells of haematopoietic origin (thymocytes) and dendritic cells. Independently, it is invaded by macrophages, also of bone marrow origin. The thymus was considered for a long time to be a component of the endocrine system, but the model of endocrine signalling failed to accurately describe the interactions between the thymic stromal network and developing T lymphocytes (thymocytes). The involution

of the thymus following hypophysectomy was the first evidence for the role of an important endocrine gland in the control of the immune system (Smith 1930). Aside from their endocrine and metabolic functions, antehypophyseal and neurohypophyseal hormones exert trophic actions on thymus and T cell development (extensively reviewed in Besedowsky & Del Rey 1996, Savino & Dardenne 2000, Berczi 2001). Despite strong evidence indicating regulation of haematopoiesis by growth hormone (GH) (Blazar *et al.* 1995, Golde & Cline 1977, Golde *et al.* 1977, Tian *et al.* 1998), it remains to be found whether these GH effects are mediated through either modulation of proliferation, activation or survival of

haematopoietic cells. Interestingly, GH administration increases thymic volume and density in HIV-infected patients (Napolitano *et al.* 2002). Moreover, GH-induced increase in thymic mass was associated with an increase in naive T cells. Combined with the fact that discontinuation of GH therapy led to the recurrence of thymic atrophy, this strongly suggests that thymopoiesis is enhanced by GH treatment (Napolitano *et al.* 2002). Although GH receptor is detected on a variety of bone marrow-derived cells, most of the effects of GH on the thymus result from a paracrine action of GH-dependent insulin-like growth factor-I (Kecha *et al.* 2000).

Involution of the thymus is observed in experimentally induced hypothyroidism (Fabris 1973, Abou-Rabia & Kendall 1994). Thymic hyperplasia and intrathymic expression of thyrotrophin receptor have been observed in patients with Graves' disease (Paschke & Geenen 1995, Murakami *et al.* 1996).

The impact of steroid hormones on thymic function has also been richly documented. Receptors for glucocorticoids, gonadal steroids and vitamin D are expressed on both thymic epithelial cells and thymocytes. Fluctuation in androgen levels leads to striking changes in thymus weight, cellularity and cell composition. The thymus of male mice is enlarged after orchidectomy or under conditions of defective androgen action (Greenstein *et al.* 1987). Androgen receptors are expressed by thymic epithelial cells and mediate androgen action on T cell development (reviewed in Olsen & Kovacs 1996, Olsen *et al.* 2001). By 1936, Selye had already observed the marked involution of the thymus and adrenal hypertrophy after severe injury (Selye 1936). Beyond the well-established pro-apoptotic effects of glucocorticoids on thymocytes (Cidlowski *et al.* 1996), the tight control of immune function and thymopoiesis by the corticotroph axis has been evidenced in physiological conditions and inflammatory disorders (reviewed in Chrousos 2000, Webster *et al.* 2002).

Physiology of the thymus

The prominent role of the thymus in T cell generation (thymopoiesis) was demonstrated by pioneering studies conducted by Miller in the early 1960s (Miller 1961). The critical role of the thymus in the establishment of central T cell self-tolerance by clonal deletion (negative selection) was evidenced more recently (Kappler *et al.* 1987, Kisielow *et al.* 1988, Nossal 1994). The integration of the thymus in endocrine physiology did not completely vanish, however, and the primary lymphoid organ is crucially located at the crossroad between the major systems of cell-to-cell communication, the neuroendocrine and immune systems. Through transcription of neuroendocrine genes in the thymus stromal network and expression of cognate receptors by immature T cells, the neuroendocrine system regulates early T cell differenti-

ation (reviewed in Geenen *et al.* 1999, 2003). Glucocorticoids also are synthesised in thymic epithelium and are able to impact on thymocyte development (reviewed in Ashwell *et al.* 2000). The processing of neuroendocrine precursors in the thymus is not coupled to classical secretion but involves constitutive pathways for membrane targeting and presentation by major histocompatibility complex (MHC) proteins (Robert *et al.* 1992, Martens *et al.* 1996a, Vanneste *et al.* 1997). Neuroendocrine self-antigens derive from the processing of precursors dominantly expressed in the thymus and usually correspond to peptide sequences highly conserved during evolution. According to the theory of clonal selection, thymic MHC presentation of neuroendocrine self-antigens would be responsible for the establishment of central immune self-tolerance of neuroendocrine principles (Martens *et al.* 1996b, Geenen *et al.* 1999, 2003).

Nowadays, the very complex process of T cell generation and differentiation may be summarised as follows. From the primary sites of haematopoiesis (embryonic yolk sac, fetal liver, then bone marrow), T cell progenitors migrate into the thymus and intensively proliferate in the thymic outer cortex. Then, intimate contacts with thymic epithelial and nurse cells promote interleukin-7 synthesis and activate recombination activating genes (*RAG1* and *RAG2*) (Fugmann *et al.* 2000, Huang *et al.* 2001). *RAG*-catalysed generation of T cell diversity in the thymus occurs through random recombination of gene segments encoding the variable part of the T cell antigen receptor (TCR) α and β chains. TCR recombination by pre-T cells constitutes a pivotal event because, among the huge number of possible combinations, many of them are able to recognise self-antigens presented by thymic MHC proteins. This negative selection represents the massive deletion of T cell clones expressing self-reactive TCRs. This process is extremely powerful since, from 100 T cell progenitors, only one or two T cells will leave the thymus in a state of self-tolerance, competence and potential activity against non-self-antigens (Scollay 1992). Thus, through a continuous presentation of constant self-antigens to thymocytes that are stochastically rearranging TCR gene segments, the physiological function of the thymus is to ensure the generation of a diverse repertoire of TCRs that are self-tolerant. In addition, the thymus is responsible for the generation of antigen-specific regulatory T cells that are able to inhibit in the periphery the self-reactivity of T cells having escaped the thymus-deletion censorship (Heddon & Mason 2000).

Generation of T cell diversity in the thymus through random TCR gene recombination

Mature T cells are characterised by their TCR able to recognise specific peptides presented by MHC class I or class II molecules. In order to respond to any particular

antigen likely to be encountered, the peripheral T cell population must be highly diverse. This huge heterogeneity, which is not encoded in the germ-line, is generated during T cell differentiation in the thymus. In this organ, T cell progenitors undergo a series of developmental events that can be monitored by membrane expression of cluster differentiation (CD) antigens. During $\alpha\beta$ (and $\gamma\delta$) T cell maturation, the juxtaposition of various gene segments leads to the generation of TCRA and TCRB chains (TCRG and TCRD). The antigen-recognising variable domains of TCR α and β chains are encoded by combinations of variable (V), diversity (D), and joining (J) gene segments (TCR β chains), or V and J gene segments (TCR α chains). V(D)J recombination is initiated by the recognition of recombination signal sequences (RSSs) that flank the coding segments. RSSs consist of conserved heptamer (CACAGTG) and nonamer (ACAAAAACC) sequences separated by a less conserved spacer of 12 or 23 bp. During maturation, pre-T cells transiently express the *RAG1* and *RAG2* genes in order to fuse together V, (D) and J segments. Two successive waves of *RAG1* and *RAG2* expression coincide with TCRB and TCRA chain rearrangements. RAG-1 and RAG-2 cooperate to recognise the RSSs and introduce hair-pinned site-specific cleavages at the signal-coding borders of a given pair of coding segments. Fusion between distinct V, (D) and J segments leads to a vast array of different TCR β chain nucleotide sequences. While this contributes in generating diversified TCR α and β chains, a greater diversification is achieved by the addition of N and P nucleotides that further broaden this pool. In fact, terminal deoxynucleotide transferase randomly incorporates deoxynucleotides at the junction of segments as they rearrange (N nucleotides). Moreover, the end-loop structure resulting from dsDNA break initiation will be further cleaved, leading to the creation of 'sticky-ends' that, once filled, will result in the insertion of palindromic sequences (P nucleotides) between rearranged segments. These two events dramatically increase TCR diversity. During any rearrangement process, the DNA located between the two RSSs is circularised, resulting in the formation of an extra-chromosomal circular excision product containing the two ligated RSSs. These TCR rearrangement excision circles (TRECs) are stable, are not duplicated during mitosis, and are thus 'diluted-out' with each cell division. A common requirement for productive rearrangement of the *TCRA* locus is the deletion of the *TCRD* locus that it encompasses. Deletion of *TCRD* is an important step during T cell differentiation and is a sign of the definitive commitment of thymocytes to the $\alpha\beta$ T lineage. This deletion mainly occurs through specific rearrangement of δ Rec and Ψ Ja, leading to the generation of a specific TREC – i.e. the signal joint (sj) TREC or δ Rec- Ψ Ja TREC – that can be observed in $\sim 70\%$ of $\alpha\beta$ T cells (De Villartay *et al.* 1988, Verschuren *et al.* 1997) (Fig. 1). TRECs are stable during a long period, as they have been detected in

41-year thymectomised patients (Sempowski *et al.* 2001). A maximum of two sjTRECs can be present within one $\alpha\beta$ T cell if the corresponding rearrangement event occurs in both alleles and if the cell does not proliferate following this rearrangement. TRECs are exported from the thymus to the periphery within recent thymic emigrants (RTEs). However, it was reported that the TCR δ locus could be excised through other recombination events that will not generate an sjTREC molecule (Veschuren *et al.* 1997). Therefore, peripheral blood quantification of sjTREC frequencies leads to an underestimation of the real frequency of RTEs. Nonetheless, it provides an unequivocal way to estimate the blood concentration of RTEs and thus, evaluate the magnitude of thymic function. Thus, TREC level in the periphery reflects RTE numbers and is largely accepted as a surrogate marker for thymic function (Kong *et al.* 1999).

In a recent paper, Hazenberg *et al.* (2000) suggested that TREC concentration should be utilised carefully to estimate thymic function as it can be affected by events occurring in the periphery such as T cell proliferation. So, data of TREC quantification should be cautiously interpreted in particular conditions such as during immune reconstitution following bone marrow transplantation or during HIV infection. However, in healthy individuals, only homeostatic naive T cell proliferation is likely to significantly affect peripheral T cell TREC content, as antigen-induced T cell proliferation only affects memory T cells and thus does not affect the RTE population. Different techniques are employed to measure TREC concentrations, such as real-time PCR, quantitative competitive PCR (Douek *et al.* 1998), and PCR-ELISA (Al-Harthi *et al.* 2000). Also, measurement units of TREC concentration vary, including TRECs/ 10^6 peripheral blood mononuclear cells, TRECs per CD45RA⁺ T cell, TRECs/ μ g DNA of T cells, and TRECs/ 10^5 CD4⁺ T cells.

Age-related evolution of sjTREC

Since the major part of thymocyte proliferation and expansion occurs before this TREC is formed, the sjTREC or δ Rec- Ψ Ja TREC is a very appropriate choice to reflect thymopoiesis (Douek *et al.* 1998). Therefore, quantification of the sjTREC frequency was selected in our studies. A two-step multiplex real-time PCR was used to quantify sjTRECs in human individuals. In order to carefully quantify the sjTREC frequency, a multiplex nested real-time quantitative PCR was performed on blood samples. In the first step, both the sjTREC molecule and a fragment of the CD3 γ chain were amplified, allowing cell quantification in the sample as described by J-F Poulin, M Sylvestre, P Champagne, M-L Dion, N Kettaf, A Dumont, M Lainesse, P Fontaine, D-C Roy, C Perreault, R-P Sékaly & R Cheynier (unpublished observations). In order to stay in the logarithmic linear phase of

Figure 1 Schematic model of recombination processes leading to sjTREC formation. *TCRD* deleting elements, $\delta\text{Rec-}\Psi\text{J}\alpha$, flank the whole *TCRD* locus. The $\delta\text{Rec-}\Psi\text{J}\alpha$ rearrangement deletes the *TCRD* genes and forms an sjTREC (also called $\delta\text{Rec-}\Psi\text{J}\alpha$ TREC) as it is generated from RAG action by the ligation of the RSS sequences flanking $\delta\text{Rec-}\Psi\text{J}\alpha$ gene segments. The $\delta\text{Rec-}\Psi\text{J}\alpha$ sequences remaining in the recombined genome are excised later in most of $\alpha\beta$ T cells during the TCR α chain recombination, and will be found in a $\text{V}\alpha\text{-J}\alpha$ coding joint (cj) TREC (Douek *et al.* 1998).

the PCR for both amplicons, the first step of amplification was only performed for 22 cycles. The second part of the nested amplification was performed using LightCycler technology (Roche), allowing the quantification of both the sjTREC and the number of cells (as half of the $\text{CD}3\gamma$ copy number) initially present in each sample. These quantifications were based on the amplification of a plasmid reference standard containing both sjTREC and $\text{CD}3\gamma$ templates. For each sample, a precise number of sjTREC copies/100 000 cells can be deduced.

Through this methodology, sjTREC concentration was determined in 41 subjects ranging from 1 to 83 years old. As shown in Fig. 2, sjTREC concentration exponentially decreases with a half-life of 19.62 years. In the population sample, no significant difference appears in the number of sjTRECs/100 000 cells between the 1–10 year (483 ± 108 s.e.m., $n=8$) and the 11–40 year groups (354 ± 77 , $n=16$) (Fig. 3). However, a very significant difference ($P<0.001$) exists between the 10–40 year and the 41–60 year groups (65 ± 27 , $n=10$). This decrease remains significant ($P<0.001$) between the 41–60 year and

the 61–80+ year (20 ± 5 , $n=7$) groups. In our hands, a correlation with gender was not found although such a correlation was previously reported (Pido-Lopez *et al.* 2001).

Thymic function in clinical conditions and in the elderly

In T cell depleting diseases such as HIV infection, treatment with highly active anti-retroviral therapy (HAART) induces a rapid increase in the numbers of detectable TRECs in peripheral cells (Douek *et al.* 1998, Poulin & Sekaly 1999, Poulin *et al.* 1999). Thus, the adult thymus retains a significant thymopoietic function and still generates naive, newly differentiated, and functional T cells for export to the periphery (Jamieson *et al.* 1999). Increases in naive T cells in HIV-infected and HAART-treated patients who had been thymectomised before suggest that naive T cells may replicate in the periphery (Haynes *et al.* 1999). Also in immuno-haematologic diseases and

Figure 2 Age-related evolution of sjTREC number per 100 000 T cells in normal subjects ($n=41$). The number of sjTRECs was measured by real-time PCR and normalised by cell number estimated with genomic CD3 γ content measured by real-time PCR of the same sample. sjTREC concentration decreases with age in an exponential way ($P=0.0014$).

Figure 3 Number of sjTRECs per 100 000 T cells in different age classes. Statistical significance of differences has been determined with Student's t -test ($***P<0.001$).

malignancies, rapid T cell regeneration after high-dose chemotherapy requires a residual thymic function (Mackall *et al.* 1995). An efficient thymic function was shown to be associated with a favourable immune reconstitution after cord blood stem cell transplantation (Talven-saari *et al.* 2002). In patients with severe combined immunodeficiency, stem-cell transplantation (Patel *et al.* 2000) or gene therapy (Hacein-Bey-Abina *et al.* 2002) was followed by an increase from undetected levels in the number of T cells containing sjTRECs. Some thymic dysfunction in T cell generation was also recently suggested in rheumatoid arthritis (Koetz *et al.* 2000).

For a long time it has been assumed that the thymic function gradually decreases after puberty, in parallel with the progressive enrichment of the thymic stroma in adipose cells (thymic adipose 'involution'). By 18 years of

age, the periphery was thought to be seeded with a complete repertoire of antigen-reactive T cells (Simpson *et al.* 1975). In recent years, more and more data have contradicted this quite dogmatic view and provided demonstrative evidence that the adult thymus remains active late in life and generates functional T cells for the peripheral lymphoid repertoire (Aspinall *et al.* 2002). In accord with those studies, as shown in Fig. 3, a significant number of sjTRECs are measured even in adults between 61 and more than 80 years of age. The persistence of thymopoiesis and generation of TCR diversity has important implications for the problem of immunosenescence. Indeed, ageing of the immune system is associated with increased morbidity and mortality from infectious diseases, decreased tumoral immunity with higher prevalence of cancers, as well as a decrease of immune self-tolerance leading to some autoimmune diseases (Pawelec *et al.* 2002). The restoration of thymic function in ageing and in various disorders seems thus to be an important objective in the elderly, in AIDS and in a series of haemato-oncological diseases. Since the thymic stroma expresses receptors for a number of hormones (reviewed in Kelley *et al.* 1986, Savino & Dardenne 2000, Murphy & Longo 2000), ensuring an adequate endocrine equilibrium should be important for maintaining thymic functions, i.e. generation of T cells with a diverse but self-tolerant TCR repertoire. Obviously, quantification of thymic T cell generation through TREC methodology now constitutes a very important parameter to measure in studies aiming to evaluate the consequence of neuroendocrine ageing (Lamberts *et al.* 1997) and diseases upon immune function, as well as the benefits expected to follow restoration of hormonal equilibrium.

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