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A normal T cell receptor beta CDR3 length distribution in patients with APECED

Heikki J. Niemi [a], Sini Laakso [a], Jukka T. Salminen [b], T. Petteri Arstila [a], Anni Tuulasvaara [a,*]

[a] Haartman Institute, Department of Bacteriology and Immunology, and Research Programs Unit, Immunobiology, University of Helsinki, PB 21, FIN-00014 University of Helsinki, Finland
[b] Department of Surgery, Hospital for Children and Adolescents, Helsinki University Hospital, PB 281, FIN-00029 HUS, Finland

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ABSTRACT

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is caused by mutations in the AIRE gene. Murine studies suggest that AIRE controls thymic expression of tissue-restricted antigens, its absence allowing nonselected autoreactive cells to escape. We tested this in humans using the TCRβ CDR3 length repertoire as a surrogate of thymic selection, as it shortens during the process. Analysis of healthy thymuses showed an altogether 1.9 base pair shortening, starting at the $CD4^+CD8^+CD3^{low}$ stage and continuing until the $CD4^+$ subset, likely encompassing both the positive and negative selection. Comparison of five APECED patients with eight healthy controls showed a skewed repertoire with oligoclonal expansions in the patients' $CD4^+$ and $CD8^+$ populations. The average CDR3 length, however, was normal and unaffected by the skewing. This was also true of the hypothesized autoreactive $CD8^+CD45RA^+$ population. We failed to detect a subset with an abnormally long CDR3 repertoire, as would be predicted by a failure in selection.

1. Introduction

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a monogenic human autoimmune disease caused by loss-of-function mutations in the autoimmune regulator (AIRE) gene [1–3]. The most common manifestations are chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal insufficiency, but most patients also have other, variable clinical manifestations [4,5]. The clinical components are usually associated with autoantibodies against the tissues in question, and the patients also have anti-cytokine antibodies [6–8].

AIRE is a transcriptional regulator with the highest expression in the medullary thymic epithelial cells (mTEC) [9,10], and therefore APECED is likely to be a result of disrupted thymic T cell development. The pathogenetic mechanism, however, remains unclear. The original theory, based largely on studies in transgenic Aire-deficient mice, holds that Aire controls ectopic expression of tissue-specific self-antigens [11] in mTECs [12,13]. These antigens are then presented to developing T cells to facilitate negative selection. In the absence of Aire negative selection is impaired, allowing the maturation and escape of autoreactive T cells [9,14]. In this view the patients should have an identifiable clonal population of pathogenic T cells, which has not been properly selected in the thymus.

The main alternative posits that AIRE is primarily involved in mTEC homeostasis [15]. Thus AIRE-deficiency would in the first place disrupt mTEC maturation or function, and absence of TSA expression in the mTECs would be a secondary and not necessarily the most important consequence. In support of this view, several studies have shown that in Aire-deficient mice mTECs are abnormal and thymic architecture may be disrupted [16–18]. Also, patients with thymoma, whose thymic stroma is profoundly abnormal and often lacks AIRE, develop similar anti-cytokine antibodies as APECED patients, suggesting shared pathogenetic phenomena in the thymus [6,19–21]. Kisand et al. have further suggested that the AIRE deficient environment causes the thymus to form tertiary lymphoid tissue and export already activated autoreactive cells [6]. Recent findings showing signs of activation already in the $CD31^+$ recent thymic emigrant population provide support for this suggestion [22].

Abbreviations: AIRE, autoimmune regulator; APECED, autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy; CDR3, complementarity determining region 3; TCR, T cell receptor; TSA, tissue-specific self-antigen; DP, $CD4^+$ $CD8^+$ double positive; SP, single positive; DP_{neg}, $CD4^+CD8^+CD3^-$; DP_{low}, $CD4^+CD8^+CD3_{low}$; DP_{high}, $CD4^+CD8^+CD3_{high}$; mTEC, medullary thymic epithelial cell.

* Corresponding author. Tel.: +358 919126892; fax: +358 2941 26382.
E-mail address: anni.tuulasvaara@helsinki.fi (A. Tuulasvaara).

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The inaccessibility of thymus in APECED patients, and differences in the phenotype between the patients and Aire-deficient mice have complicated the assessment of these competing theories of the pathogenesis of APECED. To address this question a proxy for thymic events is needed, one that can be analyzed in the circulating T cell population. Here, we have used as a proxy for thymus selection the shortening of the average length of the junctional T cell receptor (TCR) complementarity determining region 3 (CDR3) in the TCR beta chain. Previous studies have shown that a significant shift to shorter CDR3 usage takes place in the course of normal thymocyte development [23–25]. It occurs between the $CD4^+$ $CD8^+$ double positive (DP) and both $CD4^+$ single positive [7] and $CD8^+$ SP stages, and is observed also when thymocytes are compared with peripheral blood lymphocytes. Studies in mice have demonstrated that the extent of shortening is influenced by MHC haplotypes and individual Vβ segments [25–27], strongly suggesting that it is linked to intrathymic TCR-dependent selection steps. Our results confirm the thymic shortening, but identify no mature T cell subset in APECED patients which would have an abnormally long CDR3 profile.

2. Materials and methods

2.1. Patient samples

The patient group consisted of five APECED patients with a mean age of 44.8 (range 32–56); four were women. The control group included eight healthy subjects with mean age of 42.75 (range 29–55, five women). Thymic tissue was obtained from two children undergoing corrective cardiac surgery. Both children were 0.3 years old boys. The ethics committee of Helsinki University Hospital has approved this study, and informed consent was obtained from the patients or parents of the children.

2.2. Cell isolation, separation, flow cytometry and sorting

Peripheral blood mononuclear cells were extracted using Ficoll-Paque (GE Healthcare, Waukesha, WI, USA) gradient centrifugation. Thymocytes were isolated from tissue samples by mechanical homogenization. Cell sorting was performed using the FACSAria instrument (Beckton Dickinson, San Jose, CA, USA). Lymphocytes were gated in the FSC-SSC plot. After that cells were gated by CD4 and CD8 expression to DP and CD4 SP populations, and further according to CD3 expression, DP thymocytes to CD3 negative, CD3 low, and CD3 high populations, and CD4 cells to CD3 high population. The CD3 expression pattern has been previously published [28]. Purity of the isolated populations was checked with FACScan instrument (Beckton Dickinson), and was at least 89% in all populations. The sorted populations were all adjusted to the same cell number (1.4×10^6). All other flow cytometry was performed using the Cyan ADP instrument (Beckman Coulter, Brea, CA, USA). The following direct fluorochrome conjugated antibodies were used: CD3-FITC, CD4-PECy5, CD8-PE (all from Becton Dickinson). Immunomagnetic cell separation was performed using antibody-coated Dynabeads according to the manufacturer's instructions. CD8 lymphocytes and $CD8^+CD45RA^-$ and $CD8^+CD45RO^-$ subpopulations were separated as previously described [22]. CD4 lymphocytes were separated using CD4 antibody-coated Dynabeads (Invitrogen, Carlsbad, CA, USA).

2.3. TCR repertoire spectratyping

Cells were lysed with TriPure Isolation Reagent (Roche, Basel Switzerland) and total RNA was isolated using RNeasy MiniKit columns (Qiagen, Crawley, UK). First-strand cDNA was synthesized using oligo-dT-primer (Sigma-Aldrich St. Louis, MO, USA) and AMV-reverse transcriptase (Finnzymes, Helsinki, Finland). Quality of cDNA was checked using quantitative PCR and commercially available beta-actin primer-probe set (Applied Biosystems, Foster City, CA, USA). Spectratyping was done as previously described [29]. In short, PCR was performed with TCR Vβ gene and Cβ gene specific primers for 40 cycles, followed by a run-off reaction for 15 cycles using an internal, FAM-labeled Cβ gene primer. Primer sequences have been published previously [30,31] and the primers were purchased from Sigma–Aldrich. The PCR products were analyzed using capillary electrophoresis using the ABI-PRISM 3730 DNA analyzer (Applied Biosystems).

2.4. Complementarity determining region 3 analysis

Average CDR3β lengths were calculated as weighted means. The boundaries of CDR3 were defined as encompassing residues 95–106 [32]. The peak lengths and areas were extracted from spectratyping data using GeneMapper or Peak Scanner software (Applied Biosystems), and for each peak its area was divided by the sum of the areas of all peaks and multiplied by the length of the peak. These values were summed to give the weighted mean. Comparison of spectratyping profiles to a naive polyclonal cord blood repertoire was done as previously described [22]. Briefly, the relative size of each peak in the repertoire was compared to the corresponding one in the cord blood samples and the differences were calculated. The difference was put within the range of 0–100%, with 100% indicating that the repertoires had no overlap.

2.5. Statistics

Statistical analysis was performed using the IBM SPSS Statistics software (IBM, Armonk, New York, USA). *P*-values were measured using Student's paired two-tailed *t*-test, with the limit of statistical significance being $p < 0.05$. The data are shown as mean ± SD.

3. Results

3.1. Average CDR3β length of thymocytes shortens during normal development in healthy individuals

We first quantified in detail the changes occurring in the CDR3β length during normal T cell maturation in the thymus. Thymocytes were sorted according to their expression of CD4, CD8, and CD3. The earliest subset, $CD4^+CD8^+$ double positive cells lacking CD3 (hereafter DP_{neg}) do not express TCR and therefore are not yet subject to negative and positive selection. $CD4^+CD8^+CD3_{low}$ cells (DP_{low}) are the first to have a surface αβ TCR and undergo TCR-dependent selection. The shift to $CD4^+CD8^+CD3_{high}$ (DP_{high}) cells coincides with positive selection [33,34], while negative selection mostly affects cells in the thymic medulla, the $CD4^+$ or $CD8^+$ single positive thymocytes [35].

Spectratyping analysis of two normal thymuses using a panel of 22 Vβ gene family-specific primers showed a previously unreported, slight but significant increase in the CDR3β length between the earliest DP_{neg} and DP_{low} stages ($p < 0.05$). Thereafter, significant CDR3 shortening occurred both in the DP_{low} to DP_{high} (mean shortening 1.5 ± 0.6 base pairs, bp, $p < 0.0001$) and in the DP_{high} to $CD4^+$ SP shift (mean shortening 0.6 ± 0.5 bp, $p < 0.0001$, average lengths of all Vβ genes are shown in Fig. 1). Overall, the $CD4^+$ SP thymocytes expressed a 1.9 ± 0.5 bp shorter CDR3β than the nonselected DP_{neg} thymocytes did. We did not have blood samples available from the pediatric patients, but comparison with circulating $CD4^+$