Decreased recent thymus emigrant number in rheumatoid factor-negative polyarticular juvenile idiopathic arthritis

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Abstract

Objectives

To determine TCR excision circle (TREC) levels, a marker of recent thymic emigrants, in the peripheral lymphocyte pool of rheumatoid factor-negative (RF∅) polyarticular juvenile idiopathic arthritis (JIA) children.

Materials and methods

We studied TREC levels in peripheral blood mononuclear cells (PBMC) in 30 RF∅ polyarticular JIA children with active disease and in 30 age- and gender-matched healthy controls. Signal-joint TREC concentration was determined by real-time quantitative-PCR as the number of TREC copies/μg PBMC DNA gauged by a standard curve with known number of TREC-containing plasmids.

Results

TREC levels in PBMC were significantly lower in JIA ($4.90\pm3.86 \times 10^4$ TRECs/μg DNA) as compared to controls ($10.45\pm8.45 \times 10^4$ TRECs/μg DNA, $p=0.001$). There was an inverse correlation between age and TREC levels in healthy children ($r=-0.438$, $p=0.016$) but not in JIA. No clinical association was observed between TREC levels and disease activity and use of oral steroids and methotrexate.

Conclusions

The finding of decreased PBMC TREC levels in RF∅ polyarticular JIA children is consistent with a low proportion of recent thymus emigrants. This may interfere with the equilibrium between populations of polyclonal and naïve T cells versus oligoclonal memory auto-reactive T cells and, therefore, may hinder the maintenance of immune tolerance in this disease.

Key words

Juvenile idiopathic arthritis, T lymphocyte, thymus, TREC, recent thymus emigrants

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Introduction

Juvenile idiopathic arthritis (JIA) designates a group of chronic systemic inflammatory diseases with unknown etiology and with different onset types, affecting children up to 16 years old (1). Several lines of evidence suggest that T cells play a prominent role in the pathogenesis of JIA. These include oligoclonal expansion of T cells in the synovial fluid in patients with polyarticular and olygoarticular JIA, expression of activation markers such as CD25, CD45RO, CD69, MHC class II antigens on $CD4^+$ and $CD8^+$ T cells, and the predominance of Th1 type cytokines in the synovium (2-5). In addition, JIA has been associated with multiple human leukocyte antigen (HLA) class I and II alleles as well as with some non-HLA gene polymorphisms (6-9). Altogether, this supports the concept of an ongoing antigen-driven immune response with a central role for autoreactive T cells in the pathogenesis of JIA (5).

The thymus is the primary site of T lymphocyte differentiation and development. It is fully functional at birth and undergoes progressive involution along the ageing process. The persistent influx of T cells from the thymus to the periphery is an important mechanism to regulate T cell homeostasis, to maintain T cell diversity, and to control the expansion of auto-reactive T cells (10, 11). The amount of recent thymic emigrants in the peripheral blood can be estimated by the measurement of T cell receptor excision circles (TRECs) in peripheral T cells. TRECs are circular episomal DNA fragments generated during T-cell receptor rearrangement in the thymus. TRECs are stable, do not duplicate during cell division, and therefore are diluted along cell proliferation (12). TREC levels are high in cord blood cells and in healthy newborns (13). Throughout infancy there is a slight and progressive decrease in TREC counts and this decline becomes steeper with the beginning of puberty or early adulthood; the decay in TREC counts progresses throughout life and elderly people present very low TREC counts (13-15). These findings are in accordance with histological studies showing a gradual decrease in the size of the thymus, especially after the beginning of puberty, mostly due to a decrease in the thymic epithelial tissue (16, 17). Decreased TREC counts were reported in adult rheumatoid arthritis (18, 19), and in other autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis, and myasthenia gravis (20-23). Interestingly, this issue has not been appropriately studied in autoimmune diseases in children, which are in a developmental stage in which thymopoiesis is expected to be prominent. In the present study we evaluated TREC counts in peripheral blood mononuclear cells of rheumatoid factor-negative (RF∅) polyarticular JIA children with active disease and in healthy controls matched for age and gender.

Materials and methods

Patients and healthy controls

Patients with diagnosis of RF∅ polyarticular JIA, according to the International League of Associations for Rheumatology (ILAR) criteria (24), and with active disease were consecutively selected from the pediatric rheumatology outpatient clinic at the Federal University of São Paulo (UNIFESP) Medical School Hospital and the Children's Institute, University of São Paulo. Patients should have active disease at the time of sample collection defined by: (1) presence of two or more swollen and tender joints and (2) serum C-reactive protein (CRP) higher than 0.5mg/dL and/or erythrocyte sedimentation rate (ESR) higher than 15mm at the first hour. Children Health Assessment Questionnaire (CHAQ) (25), was performed for all JIA patients at the moment of blood sampling. Controls were gender- and age-matched healthy first-degree relatives of the Rheumatology Unit personnel at UNIFESP.

Patients and controls were excluded if previously subjected to thymectomy or if positive for the presence of current or previous signs and symptoms of autoimmune or chronic inflammatory diseases such as systemic lupus erythematosus, inflammatory bowel diseases, myasthenia gravis, autoimmune thyroiditis, chronic hepatitis, acute or chronic infectious diseases and thymo-

ma. The study was conducted in compliance with the Helsinki Declaration and was approved by the ethics committee at the Federal University of São Paulo and the University of São Paulo. Written informed consent was obtained from the parents or legal guardians of the children.

Quantification of TRECs by real-time polymerase chain reaction (PCR)

Peripheral blood mononuclear cells (PBMC) were isolated from 5mL EDTA blood by density-gradient centrifugation using Ficoll-Paque Plus (Amersham Bioscience, Uppsala, Sweden). Genomic DNA was extracted using the GFX™ Genomic Blood DNA purification kit (Amersham, Piscataway, NJ) according to the manufacturer's instructions. DNA concentration in all samples was determined by ultraviolet spectrophotometry at a wavelength of 260nm. Quantification of signal-joint TREC in isolated PBMCs was performed by real-time quantitative PCR on a Rotor-gene™ 3000 system (Corbett-Research, Sydney, Australia) using the intercalating agent SYBR Green (Applied Biosystems, Foster City, CA). The PCR protocol was performed as previously established (20). Briefly, each 25µL-reaction mixture contained 100ng DNA, 500nM primers (sense 5'-CCCTTTCAACCATGCTGACA-3' and anti-sense 5'-AGGTGCCTATGCATCACCGT-3'), and 12.5µL SYBR Green PCR Master Mix reagent (Applied Biosystems, Foster City, CA). The PCR protocol included an initial run at 95°C for 10 minutes, followed by 45 cycles with 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds. Each DNA sample was run in duplicate. In the same reaction each DNA sample was also tested in duplicate for β-actin (400nM for each primer and 50ng DNA) as an amplification control. The PCR primers for β-actin were as follows: sense 5'-AAGATGACCCAGGTGAGTGG-3' and anti-sense 5'-AACGGCAGAAGAGAGAACCA-3'. A standard curve was included in every PCR reaction for absolute quantification of sjTREC/µg DNA in each sample. The TREC standard curve was established with seven 10-fold-dilution solutions ranging from 10^8 to 10^2 copies/µL of plasmids containing a sjTREC fragment. The determination of TREC copy count in each sample was derived by interpolation of the PCR cycle at which fluorescence was first significantly elevated above background (the Ct or threshold cycle) into the standard curve.

Table I. Demographic and clinical characteristics of patients with juvenile idiopathic arthritis (JIA) and controls.

	JIA (n=30)	Controls (n=30)
Female / male	18 / 12	20 /10
Current age (years) (mean±SD)	10.8 ± 4.0	9.9 ± 4.6
Age at disease onset (years) (mean±SD)	6.8 ± 3.7	–
Disease duration in years (mean±SD)	4.9 ± 3.7	–
ESR (mm at first hour) (mean±SD)	45 ± 30	–
CRP levels (mg/dL) (mean±SD)	5.11 ± 5.08	–
CHAQ (mean±SD)	1.01 ± 0.92	–
Medications		
Methotrexate	25 (73.3 %)	–
NSAIDs (naproxen and indomethacin)	21 (70 %)	–
Chloroquine diphosphate	1 (3.3 %)	–
Oral glucocorticosteroids	9 (30 %)	–
Cyclosporine	1 (3.3 %)	–
Azathioprin	1 (3.3%)	–
Leflunomide	1 (3.3 %)	–

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; CHAQ: Child Health Assessment Questionnaire; NSAID: non-steroidal anti-inflammatory drug.

Statistical analysis

Descriptive analysis is presented as figures and tables with isolated data, means, standard deviation and medians. Differences among groups were analysed by Student's *t*- and Mann-Whitney tests. Spearman's linear regression was used to correlate TREC counts with age and clinical variables. $P<0.05$ was considered significant.

Results

Clinical and demographic data of patients and controls are depicted in Table I. Thirty RF∅ polyarticular JIA patients and 30 controls were included, with no statistically significant difference in gender and age distribution ($p=0.426$, $p=0.592$, respectively). The age ranged from 3 to 18 years old in JIA patients and from 1 to 17 years old in controls. Disease duration in JIA patients ranged from 2 months to 12 years. ESR at the first hour ranged from 2 to 121mm and CRP ranged from 0.01 to 18.10mg/dL. The number of inflamed (swollen and tender) joints ranged from 2 to 29. Six patients (20%) had positive antinuclear antibodies (ANA), five with a fine speckled pattern (titer range 1/160-1/640) and one with a homogeneous pattern at 1/160. Most patients (n=25) were under methotrexate, with a mean dose of 17.9 mg/week (ranging from 7.5 to 35 mg/week). Oral prednisone was used by nine patients (mean dose of 4.6mg/day, ranging from 2 to 5mg/day). Five patients were under indomethacin and 16 were using naproxen.

TREC counts in PBMC were significantly lower in JIA (4.90±3.86 x 10^4 TRECs/µg DNA) as compared to controls (10.45±8.45 x 10^4 TRECs/µg DNA, $p=0.001$) (Fig. 1). As shown in Fig. 2, a moderate inverse correlation was observed between age and TREC counts in PBMC in normal children (r=-0.438, $p=0.016$), in conformity with the previously reported age-related decay in thymus function (14, 15). In contrast no significant correlation was observed between age and TREC counts in PBMC in JIA patients (r=-0.281, $p=0.133$). TREC counts showed no correlation with ESR (r=-0.15, $p=0.46$), CRP (r=-0.27, $p=0.285$), CHAQ (r=0.066, $p=0.74$), number of inflamed joints (r=0.255, $p=0.173$), and disease duration (r=-0.126, $p=0.51$) in the 30 JIA patients. There was no difference in TREC counts between 4 patients with recent disease onset (less than 12 months) and

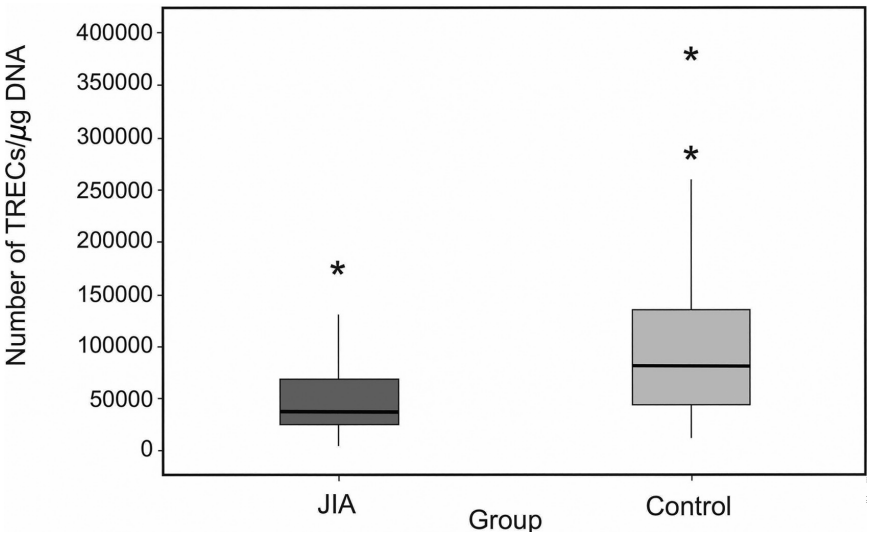

Fig. 1. Box-plot graph showing the distribution of TREC counts in peripheral blood mononuclear cells in juvenile idiopathic arthritis (JIA) patients and in age-and gender-matched healthy controls.
Footnote. Box-Plot graph: Rectangles depict 50% of the sample; thick horizontal bar corresponds to median. The symbol (*) represents outliers.

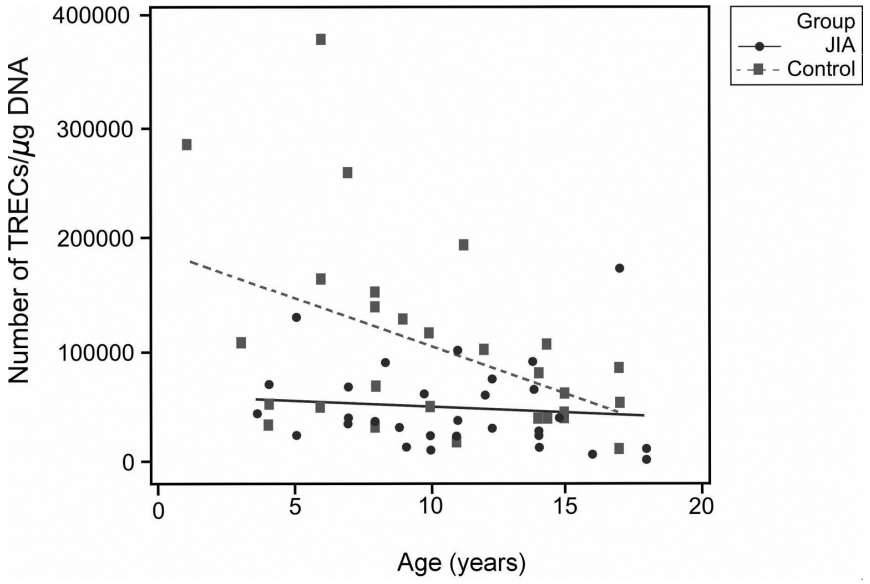

Fig. 2. Distribution of juvenile idiopathic arthritis (JIA) patients and age- and gender-matched healthy controls according to age and the number of TREC/μg DNA in peripheral blood mononuclear cells.

12 patients with long lasting disease (more than 5 years) ($3.92\pm2.14 \times 10^4$ *vs*. $5.44\pm4.68 \times 10^4$ TRECs/μg DNA, respectively, p=0.142). There was no difference in TREC counts between patients under steroids and those not using steroids ($4.82\pm3.81 \times 10^4$ *vs*. $4.93\pm3.97 \times 10^4$ TRECs/μg DNA, respectively, p=0.859) and between patients under methotrexate and those not using methotrexate ($5.05\pm4.25 \times 10^4$ *vs*. $4.50\pm2.64 \times 10^4$ TRECs/μg DNA, respectively, p=0.945). Within the group of patients under methotrexate there was no correlation between TREC counts and methotrexate dose (r=-0.355; p=0.105) or cumulative methotrexate dose (r=-0.27; p=0.236). There were only nine patients under steroids and the doses were low (maximal 5mg/day prednisone) and homogenous so that we could not perform statistical analysis between steroid dose and TREC counts.

Discussion

The present study has examined the proportion of recent thymic emigrants in the peripheral blood T cell pool in children with active RF∅ polyarticular type JIA by quantifying TREC levels in peripheral blood mononuclear cells. Peripheral blood TREC counts were significantly decreased in RF∅ polyarticular JIA children with active disease as compared to healthy age- and gender-matched controls. A moderate decrease in TREC levels with age was observed in healthy children, in agreement with the previously reported age-dependent TREC count decrease (12, 15). In contrast, no age-related TREC count decay was observed in JIA children. No correlation was observed between TREC counts and disease activity markers, disease duration, use of oral steroids, and methotrexate therapy.

Decreased TREC counts have been previously demonstrated in several adult autoimmune diseases (18-20; 26), suggesting that the disturbance in TREC dynamics is a common component in the pathophysiology of autoimmune diseases. However, the subject has been largely unexplored in children with autoimmune diseases. Recently, two studies have analysed recent thymus emigrants in JIA (27, 28). Prelog *et al*. studied 22 JIA patients with inactive disease (with oligoarticular, polyarticular, and systemic type) and observed reduced frequency of TREC in $CD4^+$ $CD45RA^+$ naive T cells as compared to controls (27). In addition these patients also presented increased telomeric erosion and increased frequency of Ki67-positive cells among the $CD4^+$ $CD45RA^+$ naive T cell population. Lorenzi *et al*. have undertaken a comprehensive exploration of recent thymus emigrants in JIA patients (8 systemic, 10 RF⊕ polyarticular, 12 RF∅ polyarticular, 27 persistent oligoarticular, 7 extended oligoarticular, 4 psoriatic, and 2 enthesitis) with disease activity status not stated (28). The authors have observed no difference in whole blood TREC counts between JIA patients and healthy controls. Although apparently discordant, these two studies and the present report provide important complementary information for the understanding of T cell pathophysiology in JIA, since they address distinct cell populations (whole

blood, PBMC, and $CD4^+$ $CD45RA^+$ naive T cells) and distinct disease subsets. In fact the term JIA encompasses several disorders with distinct pathophysiologic features and with striking differences in severity, and outcome. Aiming to study a relatively homogeneous group of patients, we have selected exclusively children in active stage of RF∅ polyarticular type JIA, a frequent subtype and consistently associated with T cell abnormalities. Our results confirm Prelog's previous findings of decreased TREC counts in JIA children and extend this observation to the active stage of the disease. The difference in results obtained by Lorenzi *et al*. may be related to the highly heterogeneous patient composition in that study, in which several subtypes of JIA were included. Taken together, the data on TREC counts presented here and in the literature reinforce the concept that JIA is a heterogeneous disorder also with respect to pathophysiology.

In infants, the thymus is a relatively large organ with 90% of its volume being represented by the thymic epithelial tissue (29). The measurement of TREC counts in peripheral lymphocytes has been introduced as a valuable index of the proportion of recent thymic emigrants in the peripheral blood T cell pool, and provides an indirect estimation of thymic T cell output. Healthy children present high levels of TREC counts in peripheral blood and, genetically athymic children with DiGeorge's Syndrome and children with severe combined immunodeficiency (SCID) present undetectable or very low TREC levels (30, 31). After transplantation of thymus tissue in children with DiGeorge's syndrome and following stem cell or bone marrow transplantion in SCID, there is a gradual increase in TREC levels in the peripheral blood, associated with a gradual reconstitution of the immune system (30-32).

The observed low TREC counts in RF∅ polyarticular JIA may be indicative of premature thymic involution in this disease. Premature thymic atrophy has been described in several autoimmune diseases and may result in considerable alteration in T cell homeostasis (33, 34). The use of immunosuppressive drugs and glucocorticosteroids is also known to be able to induce thymus atrophy and to inhibit lymphopoiesis and could presumably have contributed to the decreased TREC levels found in the present study (35). Among the 30 JIA patients herein investigated, no significant correlation was observed between TREC counts and the use of methotrexate or glucocorticosteroids. Similar findings were reported by Lorenzi *et al*. (28). Nonetheless, it should be noted that several factors apart from thymic output, are able to affect TREC levels in peripheral mononuclear cells, such as peripheral T cell proliferation, peripheral T cell death, and T cell redistribution (36). Accelerated peripheral T cell turnover can dilute out $TREC^+$ cells vis-à-vis the T cell pool. In fact increased telomeric erosion and Ki67 expression have been reported in peripheral blood T cells in JIA (27). It is conceivable, thus, that the observed decreased proportion of $TREC^+$ cells in JIA could be determined by more than one of the above mentioned factors.

Regardless of the underlying mechanisms, the decreased TREC counts observed in RF∅ polyarticular JIA children is a relevant finding per se because it indicates a low proportion of recent thymus emigrants in the peripheral T cell pool. The size and diversity of the peripheral T cell pool are homeostatically regulated and remain relatively stable over ageing mainly due to two mechanisms: the influx of recent thymic emigrants and the homeostatic peripheral proliferation of mature T cells (37). Peripheral T cell pool maintenance in the presence of low thymic export would cause gradual loss of diversity, through the attrition of naïve T cells and the expansion of the memory cell compartment. The low proportion of TREC cells herein demonstrated in RF∅ polyarticular JIA patients is consistent with a disequilibrium in the composition of the peripheral T cell pool and can further indicate decreased T cell diversity and tendency towards oligoclonal proliferation. As previously demonstrated, several autoimmune conditions including JIA are associated with T cell oligoclonality at the involved tissues (38, 39).

In conclusion, our study originally demonstrated decreased TREC counts in children with active RF∅ polyarticular JIA. This finding is in agreement with previous similar studies in several autoimmune conditions in adults. In all these conditions, the finding of decreased TREC counts is consistent with a low proportion of recent thymus emigrants in the peripheral T cell pool, what may in turn interfere with the maintenance of immune tolerance and with the suppression of auto-reactive T cell clones. This preliminary finding must be further explored in additional studies in order to formally verify the behavior of recent thymic emigrants in other JIA subtypes, possible correlation with disease activity, and possible effect of immunosuppressive therapy.

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