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PAPER

Decreased number of T cells bearing TCR rearrangement excision circles (TREC) in active recent onset systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is characterized by several T lymphocyte abnormalities. An indirect assessment of recent thymus emigrants (RTE) has been recently been made available by measuring the number of TCR recombination excision circles (TREC) in peripheral T cells. We studied TREC levels in peripheral blood mononuclear cells (PBMC) of 32 SLE patients with active disease and 32 normal age- and sex-matched controls. Signal-joint TREC concentration was determined by real-time quantitative-PCR as the number of TREC copies/μg PBMC DNA. SLE patients had lower TREC levels ($4.1 \pm 3.9 \times 10^4$ TREC/μg DNA) than controls ($8.9 \pm 7.9 \times 10^4$/μg DNA) ($P = 0.004$). There was an inverse correlation between age and TREC levels in controls ($r = -0.41$, $P = 0.02$) but not in SLE patients. No clinical association was observed between TREC levels and clinical and laboratory SLE manifestations. TREC levels tended to be lower in patients with SLEDAI above 20 than in the rest of the patients ($P = 0.08$). The decreased PBMC TREC levels is indicative of a low proportion of RTE in SLE and could be caused by decreased RTE output and/or by increased peripheral T cell proliferation in this disease. The under-representation of RTE in the peripheral T cell pool may play a role in the immune tolerance abnormalities observed in SLE. *Lupus* (2004) **13,** 906–911.

Key words: systemic lupus erythematosus; T lymphocyte; thymus; TREC

Introduction

Systemic lupus erythematosus (SLE) is considered a paradigm of systemic autoimmune disease and its pathophysiology is still not completely elucidated. Among the immune abnormalities identified in SLE, a large body of evidence points to a primary T lymphocyte dysfunction mediating B cell hyperactivity.[1,2] The thymus is the primary site of T cell maturation and development. Several studies in murine SLE models have reported on alterations in the microenvironment and function of the thymus, leading to the hypothesis of a possible involvement of this organ in the disease pathogenesis.[3,4] Histopathologic studies in distinct murine SLE models (NZB/W F_1, BXSB and MRL) have shown early thymus atrophy, most prominent in the cortical region but also affecting the medulla.[5] Specific abnormalities in the thymus microenvironment, including cortical epithelial cell-free regions, have been described in murine SLE models and shall play a crucial role in disease pathogenesis given that cortex and medulla are responsible for positive and negative selection of T cells, which results in the elimination of self-reactive cells and in the modeling of the T cell receptor (TCR) spectrum expressed by peripheral T cells.[4,6] Thymus atrophy and a series of abnormalities in peripheral T cell function have been registered in SLE patients.[1,2,7] Furthermore, association between SLE and thymoma has been reported in a frequency higher than expected by chance and thymectomy has brought diverse consequences on the course of human SLE.[8,9]

Direct assessment of thymic function in human SLE has been limited due to the lack of known appropriate markers of recent thymic emigrants. The recent availability of a means for measuring the number of TCR recombination excision circles (TREC) copies in peripheral T cells has provided a novel marker for estimating thymic T cell production.[10,11] TREC are episomal DNA circles generated during intrathymus

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T cell maturation by excision rearrangement of TCR genes that results in contiguous and functional genes responsible for the transcription of TCR-$\alpha\beta$ and TCR-$\gamma\delta$ membrane receptors. A mandatory step in $\alpha\beta$ T cell neogenesis is the excision of the TCR-δ locus from within the TCR-α locus. This process results in the generation of two episomal DNA fragments, a signal-joint TREC (sjTREC) and a coding-joint TREC (cjTREC), which can be detected in $\sim$70% of $\alpha\beta$ T cells leaving the thymus.[12] These products are relatively stable and do not undergo replication during mitosis, what means that they are diluted out with each cellular division in peripheral T cells.[10,12] TREC are found in relative high numbers of copies in peripheral T cells during the first two decades of life and decline with age.[10,13] TREC counts in peripheral blood mononuclear cells (PBMC) have been widely used to estimate the number of recent thymus emigrants (RTE) in several situations in which immune deterioration or reconstitution is evident, such as in HIV-1 infection, in the follow-up of HIV-1 patients undergoing treatment, and in the recovering stage of hematopoietic transplantation.[13,14]

Notwithstanding the evidence that immune function is compromised in SLE, the function of the thymus and the dynamics of RTE are still obscure in these patients. Considering the large body of evidence pointing to T cell involvement in human SLE, an estimate of the concentration of RTE in PBMC may provide insight into the possible role of T cell homeostasis in the immunopathogenesis of this disease. In the present study, we evaluated the number of sjTREC copies by real-time automated polymerase chain reaction (PCR) in PBMC of SLE patients with active disease and in healthy controls matched for age and gender.

Materials and methods

Patients and healthy controls

Patients meeting the American College of Rheumatology SLE criteria with active disease and disease duration not greater than three years were consecutively selected from the SLE outpatient clinic at UNIFESP Medical School Hospital.[15] All patients underwent a thorough rheumatologic examination and had anti-native DNA antibodies (indirect immunofluorescence on *Crithidia luciliae*) and total hemolytic complement (radial immunohemolysis) serum levels determined. Disease was considered active when the SLE Disease Activity Index (SLEDAI) was above 5.[16] Gender and age-matched normal controls were composed of students, nurses and physicians at UNIFESP Medical School Hospital. Patients and control individuals were excluded if previously subjected to thymectomy or if positive for the presence of current or previous symptoms and signs of autoimmune or chronic inflammatory diseases such as connective tissue diseases (except for SLE), inflammatory bowel diseases, myasthenia gravis, autoimmune thyroiditis, chronic hepatitis and thymoma. Informed consent was obtained from each participant.

Quantification of sjTREC

Peripheral venous blood samples (10 mL) were collected into EDTA. PBMC were isolated by standard Ficoll/Hypaque density gradient centrifugation. Genomic DNA from unfractionated PBMC was extracted using the GFX^{TM} 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 260 nm and adjusted to a concentration of 60 ng/μL. Signal-joint TREC concentration was determined by real-time quantitative PCR on a ABI 5700 Sequence Detection System using the intercalating agent Sybr Green (Applied Biosystems, Foster City, CA). The PCR primer sequences were as follows: sense 5′-CCCTTTCAACC ATGCTGACA-3′, antisense 5′-AGGTGCCTATGC ATC ACCGT-3′. Each 25 μL reaction mixture contained 60 ng DNA, 900 nM each primer, and 12.5 μL Sybr Green PCR Master Mix reagent (Applied Biosystems, Foster City, CA). The PCR protocol included an initial run at 50°C for 2 minutes and at 95°C for 10 minutes, followed by 45 cycles with 95°C for 15 seconds and 60°C for 1 minute. Each DNA sample was run in triplicate. In the same reaction, each DNA sample was also tested in duplicate for β-actin as an amplification control. The PCR primers for β-actin were as follows: sense 5′-TCACCCACACTGTGCCC ATCTACGA-3′, antisense 5′-CAGCGGAACCGCTC ATTGCCAATGG-3′.

A standard curve was included in every PCR reaction for absolute quantitation of TREC in each patient and control DNA sample. To generate the TREC standard curve, a 376 bp TREC fragment was cloned into the pCR II-TOPO plasmid (Invitrogen, Carlsbad, CA) (kindly provided by E. Hochberg, Harvard Medical School, Boston, Massachusetts). After amplification in DH5α *Escherichia coli*, plasmid DNA was purified with Qiagen Miniprep Kit (Qiagen, Valencia, CA) and the sequence of the cloned TREC DNA insert was confirmed by automated DNA sequencing. The number of TREC copies in the DNA preparation was estimated after determining the DNA concentration. The TREC standard curve included seven 10-fold dilution points ranging from 10^7 to 10^1 copies/μL.

The determination of the starting copy number of TREC was derived by interpolation of the PCR cycle at which fluorescence was first significantly elevated above background in each sample (the Ct or threshold cycle) against the standard curve.

Statistical analysis

Differences in age and in numbers of TREC/μg PBMC DNA between patients and normal controls were analysed by Student's *t* test. Differences between age groups and between SLEDAI score groups were analysed by the Mann-Whitney test. Numbers of TREC/μg PBMC DNA were correlated with age and SLEDAI score by Pearson's rank correlation coefficient. Spearman's linear regression was used to correlate TREC levels with clinical variables, anti-DNA serum levels, and the use of prednisone and cyclophosphamide. $P < 0.05$ was considered significant.

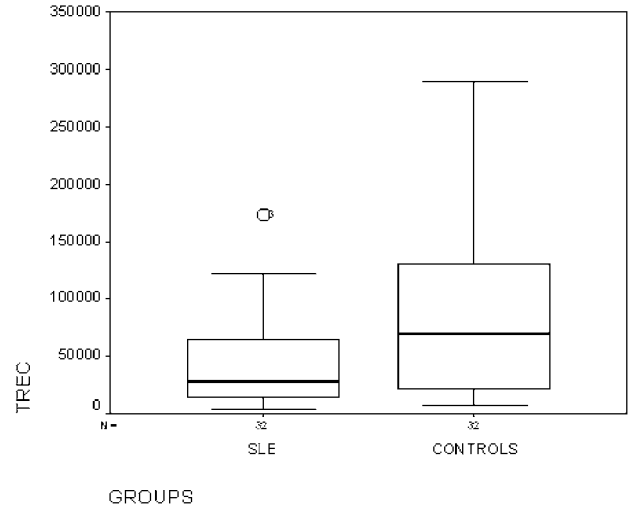

Figure 1 Distribution of normal controls and patients with SLE according to the number of TREC copies per μg PBMC DNA. Boxplot graph: rectangles depict 50% of the sample; thick horizontal bar corresponds to median; upper and lower horizontal bars represent highest and lowest figures. Symbol above the upper horizontal bar represents outlier.

Results

Thirty-two SLE patients and 32 healthy controls were analysed. The mean age and standard deviation of SLE patients was 26.7 ± 8.2 years (ranging from 18 to 47) whereas control individuals were 27.2 ± 6.3 years (ranging from 18 to 50). There was no statistically significant difference in the age between SLE patients and control individuals. In both groups there were 30 females and two males. Disease duration had a mean and standard deviation of 1.1 ± 1.1 years, ranging from one month to three years. The SLEDAI had a mean and standard deviation of 14.6 ± 5.08, ranging from 6 to 28. Table 1 depicts the frequency of SLEDAI parameters, SLEDAI intervals, and medication in use in the patient group. The most frequent clinical manifestation was kidney involvement, occurring in 23 patients (71.9%). Anti-native DNA antibodies and hypocomplementemia were detected in 50 and 34.4% of the patients, respectively. According to the classification of Cook *et al.*,[16] the majority of the patients had a high (53.1%) to very high (15.6%) degree of disease activity. Most patients (87.5%) were under oral and/or pulse steroids and three patients had a recent diagnosis and had not yet been started on any kind of therapy.

The mean TREC count in PBMC for the control individuals was $8.9 \pm 7.9 \times 10^4$/μg DNA. SLE patients had significantly lower TREC counts in PBMC ($4.1 \pm 3.9 \times 10^4$ TREC/μg DNA) compared to age-matched controls ($P = 0.004$) (Figure 1). As shown in Figure 2, there was an inverse correlation between age and TREC counts in PBMC in control individuals ($r = -0.41$, $P = 0.02$) but not in SLE patients ($r = -0.12$, $P = 0.51$). When SLE patients were stratified in two age subgroups (≤30 years and >30 years) no difference in TREC count was found between SLE patients aged up to 30 years and those older than 30 years (Table 2). Furthermore, TREC levels in control individuals older than 30 ($3.8 \pm 5.1 \times 10^4$ TREC/μg DNA) were comparable to TREC levels in SLE patients who were 18 to 30 years

Table 1 Distribution of SLE patients according to the frequency of SLEDAI parameters, SLEDAI intervals and medication in use

SLEDAI parameter	*Number of patients (%)*	*SLEDAI interval/medication*	*Number of patients (%)*
Skin lesions	10 (31.3)	SLEDAI (6–10)	10 (31.3)
Arthritis	7 (21.9)	SLEDAI (11–19)	17 (53.1)
Renal involvement	23 (71.9)	SLEDAI ≥ 20	5 (15.6)
Cytopenia	6 (18.8)	Oral prednisone	27 (84.4)
Serositis	1 (3.1)	Chloroquine phosphate	11 (34.4)
Neuropsychiatric manifestation	4 (12.5)	Azathioprin	2 (6.25)
Hypocomplementemia	11 (34.4)	Cyclophosphamide IV pulse	8 (25)
Anti-DNA antibodies	16 (50)	Methylprednisolone IV pulse	5 (15.6)

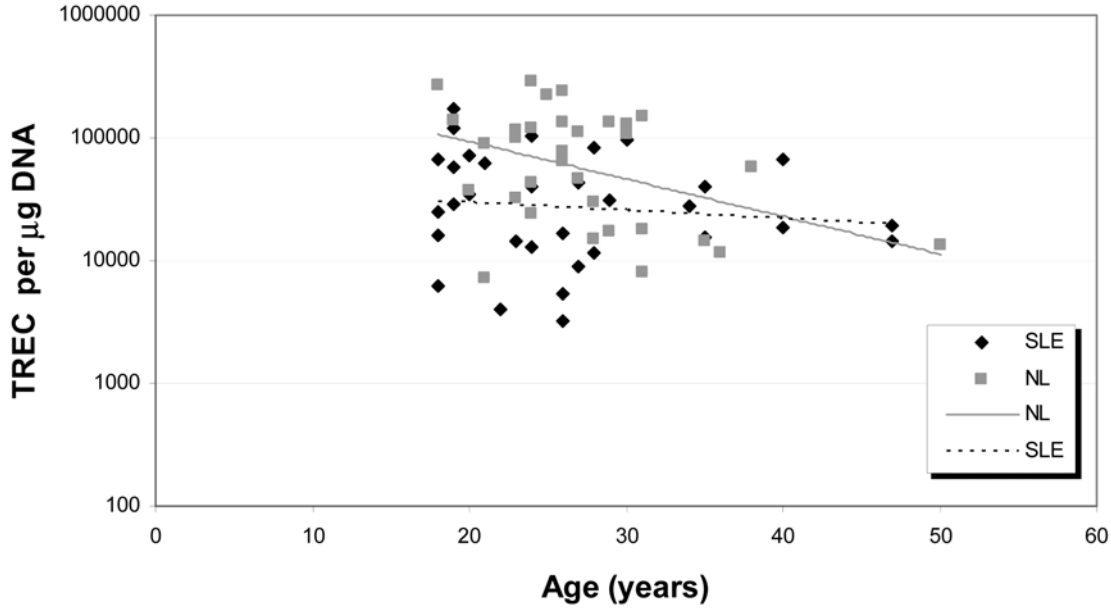

Figure 2 Distribution of SLE patients and healthy controls (NL) according to age and the number of TREC copies per μg PBMC DNA. TREC per μg PBMC DNA was lower in SLE patients (◆) than in healthy controls (■) ($P = 0.004$). TREC counts decreased with age in healthy controls ($r = -0.41$, $P = 0.02$), but not in SLE patients ($r = -0.12$, $P = 0.51$).

old ($4.5 \pm 4.3 \times 10^4$ TREC/μg DNA) ($P = 0.56$). Finally, TREC levels in SLE patients above 30 years old did not differ from controls above 30, contrasting with the significant difference between SLE patients who were 18 to 30 years old and age-matched controls ($P = 0.002$) (Table 2). There was no statistically significant correlation between SLEDAI and TREC levels in the 32 SLE patients ($r = -0.07$, $P = 0.68$). However, there was a trend for lower TREC counts in the five patients with SLEDAI ≥ 20 ($1.7 \pm 1.5 \times 10^4$ TREC/μg DNA) as compared with patients with SLEDAI < 20 ($4.6 \pm 4.1 \times 10^4$ TREC/μg DNA) ($P = 0.08$). According to Table 3, there was no association between TREC counts and the presence of anti-native DNA antibodies or distinct organ involvement. Use of steroids or cyclophosphamide was also not associated with difference in TREC counts (Table 3). In two of the three SLE patients under no treatment, TREC counts were very low: patient P4 (29 years and SLEDAI 10) had 3.1×10^4 TREC/μg DNA; and patient P17 (26 years and SLEDAI 10) had 5.3×10^3 TREC/μg DNA. However, the third one of them had high counts: patient P23 (24 years and SLEDAI 13) had 1.0×10^5 TREC/μg DNA.

Table 2 TREC levels per μg PBMC DNA according to age in healthy controls and in SLE patients

Age levels	*Number of TREC copies per μg PBMC DNA (mean ± standard deviation)*	
	Healthy controls	*SLE*
18–30 years	$10.0 \pm 8.1 \times 10^4$/μg DNA[a]	$4.5 \pm 4.3 \times 10^4$/μg DNA[b]
>30 years	$3.8 \pm 5.1 \times 10^4$/μg DNA[c]	$2.9 \pm 1.9 \times 10^4$/μg DNA[d]

Mann-Whitney test: a × b: $P = 0.002$; c × d: $P > 0.05$; a × c: $P = 0.02$; b × d: $P > 0.05$.

Table 3 Correlations of TREC levels per μg PBMC DNA with clinical manifestations and use of glucocorticosteroids or cyclophosphamide in the 32 SLE patients.

Variáveis	r^a	P
Skin lesions	0.02	0.94
Serositis	−0.22	0.22
Neuropsychiatric manifestation	−0.16	0.37
Renal manifestation	0.18	0.33
Cytopenias	−0.04	0.85
Vasculitis	−0.05	0.78
Anti-native DNA	−0.21	0.24
Use of oral prednisone	0.14	0.46
Cyclophosphamide IV pulse	0.07	0.70
Methylprednisolone IV pulse	0.12	0.53

[a]Spearman's correlation coefficient by rank.

Discussion

This is the first report on TREC quantitation in PBMC of patients with idiopathic SLE. This parameter provides an estimate of the number of T cells that have not undergone post-thymus clonal expansion and probably reflects closely the RTE population.[17] The frequency of RTE in PBMC of patients with

active SLE was assessed by accurate determination of TREC counts by real-time quantitative PCR. TREC counts in PBMC in patients with active SLE were significantly decreased in relation to healthy age- and gender-matched controls. TREC counts showed an inverse correlation with age in the normal control group but not in patients with SLE. There was no association between TREC counts and the presence of anti-DNA antibodies and specific organ involvement. SLEDAI did not show correlation with TREC levels in the whole group of patients.

Low TREC counts were present early in the course of clinically evident disease since all subjects had no more than three years of disease and 19 of the 32 patients had clinical presentation within the last year. While this observation may suggest that RTE abnormalities represent an early event in the disease, they do not necessarily parallel the earliest immune abnormalities underlying the development of SLE, which may be present years before clinical SLE.[18] Control individuals presented the expected age-dependent fall in TREC counts as reported previously.[10,13] In contrast, there was no significant age-dependent fall in TREC counts in SLE patients. Accordingly, the difference in TREC counts between control individuals and SLE patients was statistically significant only for individuals under 30 years of age. The reason for this observation is not clear at the moment and further evaluation with an extended sample of older SLE patients is warranted. The absence of statistically significant correlation with SLEDAI might have been favored by the narrow SLEDAI intervals of the sample since all patients had active disease. In fact, the few patients with the most extreme SLEDAI score showed a trend for lower TREC counts ($P = 0.08$). This finding deserves further investigation with a larger sample including patients with inactive disease.

Low TREC counts have been reported previously in other autoimmune diseases, such as multiple sclerosis, myasthenia gravis and rheumatoid arthritis, suggesting that this abnormality may be common to several autoimmune processes.[19–21] On the other hand, patients with procainamide-induced lupus showed TREC counts similar to procainamide users with no evidence of autoimmunity. However, there was a statistically significant correlation between TREC counts and serum anti-chromatin IgG antibody activity in patients with drug-induced lupus.[22]

The exact interpretation of the decreased TREC counts in SLE is not readily evident. SLE is a complex autoimmune disease with several immune abnormalities that could ultimately lead to decreased peripheral TREC counts. Peripheral TREC counts are affected mainly by thymus output, peripheral T cell division rate and peripheral T cell death rate.[23] In HIV patients, the low TREC counts in T cells have been shown to be related both to decreased thymus output and to an increased peripheral T cell proliferation rate.[13,24] The observed low TREC counts in SLE may be indicative of a low production of T cells by the thymus in this disease. This finding is supported by the reported associations between SLE and thymus abnormalities such as early thymus atrophy and histopathologic changes in the thymus of murine SLE models and in human SLE.[5–9] On the other hand, the presence of TREC, although in low counts, shows clearly that the thymus does keep some degree of activity in SLE patients. Alternatively the low TREC counts may be due to increased peripheral proliferation of T cells, which would dilute TREC counts. Several pieces of evidence point to an increased T cell activation and proliferation rate in SLE, including increased T cell expression of surface HLA-DR/DP molecules and IL-2 receptors, increased serum levels of IL-2, soluble IL-2 and TNF-α receptors, shortened telomeres and increased telomerase activity in peripheral lymphocytes.[25–27] Finally, some studies have reported an increased peripheral lymphocyte death rate in SLE, which might also be involved in the present findings.[28]

Another putative factor influencing RTE frequency is the use of immunosuppressive drugs and glucocorticosteroids, known to be able to induce thymus atrophy and to inhibit lymphopoiesis.[29] Among the 32 patients in the present study, 28 were under oral and/or IV glucocorticosteroids and eight were receiving IV cyclophosphamide pulse therapy. No correlation was observed between TREC counts and the use of glucocorticosteroids or cyclophosphamide. Indeed, it should be noticed that the only three patients under no treatment presented TREC counts comparable to the other patients. It is relevant that TREC counts have been shown previously not to be affected by the use of glucocorticosteroids, azathioprine or mycophenolate mofetil as well as cyclosporin.[17,19]

The immunologic features mentioned above are compatible with the hypothesis that the low TREC counts observed in SLE patients might be caused by a combination of varied degrees of decreased RTE thymus output, increased peripheral T cell proliferation and increased peripheral lymphocyte death rate. Irrespective of the individual contribution of each of these three factors, the low proportion of RTE in the peripheral T cell pool is a remarkable finding *per se* and may have consequences to the immune tolerance abnormalities observed in SLE. The complex and dynamic homeostasis of the peripheral T cell pool requires a constant input of novel T lymphocytes. Although peripheral T cell proliferation does contribute to the input of novel T lymphocytes, the TCR repertoire derived from these is likely to be

heavily biased by the environmental exposure, including the exposure to self-antigens. It has been suggested that one important mechanism for controlling the expansion of auto-reactive T cells in the periphery would be the continuous input of naive T cells by the thymus. These RTE would counterbalance the antigen-biased proliferation of peripheral T cells and therefore contribute to the homeostasis of the TCR repertoire.[30,31] The present demonstration of low TREC counts in PBMC in patients with active SLE is indicative of a low representation of RTE in the pool of peripheral T cells, which might be associated with an increased expression of auto-reactive T cells in this disease. Future studies shall address the impact of T cell peripheral proliferation, thymopoiesis, and peripheral T cell death in determining low TREC counts in SLE and shall investigate the role of this abnormality in SLE pathophysiology.

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