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ORIGINAL ARTICLE

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Decreased numbers of signal-joint T cell receptor excision circle-containing CD4+ and CD8+ cells in systemic lupus erythematosus patients

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Abstract Systemic lupus erythematosus (SLE) patients have a decreased number of peripheral blood T cells containing signal-joint T cell receptor excision circles (Sj TRECs), which are considered an indicator of thymic output. The objective of this study was to investigate the mechanism of the decrease in such T cells. Peripheral blood T cells from SLE patients were classified into CD4+ and CD8+ cells. Sj TREC levels were measured by real-time PCR. Telomerase activity was determined by the telomeric repeat amplification protocol assay. The numbers of Sj TREC containing CD4+ and CD8+ cells were lower in the peripheral blood of SLE patients than in the controls. A correlation was found between the numbers of Sj TREC-positive CD4+ and CD8+ cells. The level of TRECs is influenced by an increase in cell division. To examine this increase, telomerase activity as an indicator of cell division was measured simultaneously; however, there was no correlation between the Sj TREC level and telomerase activity. These results suggest that decreased thymic output occurs in SLE patients.

Key words CD4 · CD8 · SLE · Telomerase · T lymphocyte · T cell receptor excision circles (TRECs)

Introduction

During differentiation in the thymus, T cells undergo T cell receptor (TCR) gene rearrangement. Genes determining the variable region of the TCR gene belong to the V, D, or J region, and a gene in each region is selected and joined together to generate a diversity of active genes. The juxtaposition of given genes in these regions results in the looping out of the segment between two genes, and the loop segment is excised from the genes in the form of a closed circle, which is called a TCR recombination excision circle (TREC).[1] Among these, the signal-joint TREC (Sj TREC) is formed immediately before T cells leave the thymus.[1] Thus, the number of Sj TREC-containing peripheral blood T cells is considered to be an indicator of recent thymic output.[1] On the other hand, the TREC level in peripheral blood T cells is known to be influenced by an increase in the rate of T cell division.[1,2]

Recently, Kayser et al.[3] have reported that systemic lupus erythematosus (SLE) patients show lower peripheral blood mononuclear cell (PBMC) TREC levels than controls. They speculated that the abnormal TREC levels could be caused by decreased thymic output or increased peri pheral T cell division.

Telomerase activity is known as an indicator of increased lymphocyte division.[4] We reported that peripheral lymphocytes from SLE patients had a high telomerase activity, suggesting the active division of peripheral blood lymphocytes from SLE patients.[5,6] Therefore, in this study, to investigate the detailed mechanism of abnormalities in the levels of TRECs of PBMC from SLE patients, we classified peripheral blood T cells from SLE patients into CD4+ and CD8+ cell fractions, measured TREC levels in each cell fraction, and examined the relationship between TREC levels and telomerase activity.

Materials and methods

Patients

The 21 SLE patients whose Sj TREC levels were measured were all women, ranging from 25 to 50 years of age (mean 35.4 ± 8.6 years). As controls, 30 healthy individuals (1 man and 29 women) whose age ranged from 21 to 53 years (mean 34.0 ± 9.6 years) were analyzed. Sj TREC levels and telomerase activity were simultaneously measured in 15 of the SLE patients. They were all women ranging from 25 to 50

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years of age (mean 35.7 ± 9.3 years). Before the start of this study, the SLE patients and healthy subjects received an explanation about this study, and after written informed consent was obtained, about 20 ml of heparinized peripheral blood was collected.

Isolation of CD4+ and CD8+ cells

The PBMC fraction was isolated by Ficoll density gradient centrifugation, and purified into CD4+ and CD8+ cells using an MACS system (Miltenyi Biotec, Gladbach, Germany). The CD4+ and CD8+ cells obtained were more than 95% pure by FACS analysis using a FACSCalibur (Becton Dickinson, San Jose, CA, USA).

Measurement of Sj TREC levels

DNA was extracted from CD4+ and CD8+ cells using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Sj TREC levels were determined by real-time quantitative PCR using an ABI Prism 7700 (Applied Biosystems, Foster City, CA, USA) according to the method of Hazenberg et al.[7] The sequences of the forward and reverse primers and probe for the Sj TREC were 5′-CCATGCTGACACCTCTGGTT-3′, 5′-TCGTGAGAACGGTGAATGAAG-3′, and 5′-CACGGTGATGCATAGGCACCTGC-3′, respectively. As an internal control, the expression of the TCR α-chain constant region was simultaneously analyzed using the following forward and reverse primers and probe: forward primer, 5′-CCTGATCCTCTTGTCCCACAG-3′; reverse primer, 5′-GGATTTAGAGTCTCTCAGCTGGTACA-3′; and probe, 5′-ATCCAGAACCCTGACCCTGCCG-3′. The thermal cycling conditions for the Prism 7700 (Applied Biosystems) were as follows: 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C, and 1 min at 60°C. To generate a standard curve for real-time PCR, the Sj TREC and TCR genes were inserted into PCR II TOPO plasmids using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), and serial dilutions of these plasmids were amplified by PCR. The percentage of the number of copies of the Sj TREC gene to that of the TCR gene was defined as the Sj TREC level.

Measurement of telomerase activity

Telomerase activity was measured by a telomeric repeat amplification protocol (TRAP) assay using a TRAPeze Telomerase Detection Kit (INTERGEN, Purchase, NY, USA) and a modification of the protocol. Each sample (1.0×10^5 cells) was suspended in 100 µl of 1× CHAPS lysis buffer, homogenized, and incubated on ice for 30 min. The cell lysate was centrifuged at 15000 rpm at 4°C for 20 min. To

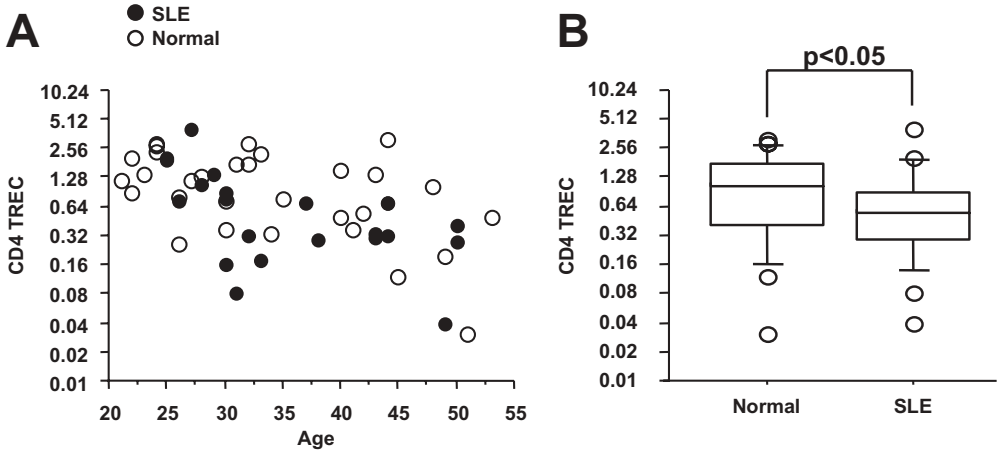

Fig. 1. Signal-joint T cell receptor excision circle (Sj TREC) levels of CD4+ cells in healthy individuals and systemic lupus erythematosus (SLE) patients. **A** Age distribution of Sj TREC levels of CD4+ cells. **B** Sj TREC levels of CD4+ cells in healthy individuals and SLE patients. Sj TREC levels are expressed on a log2 scale

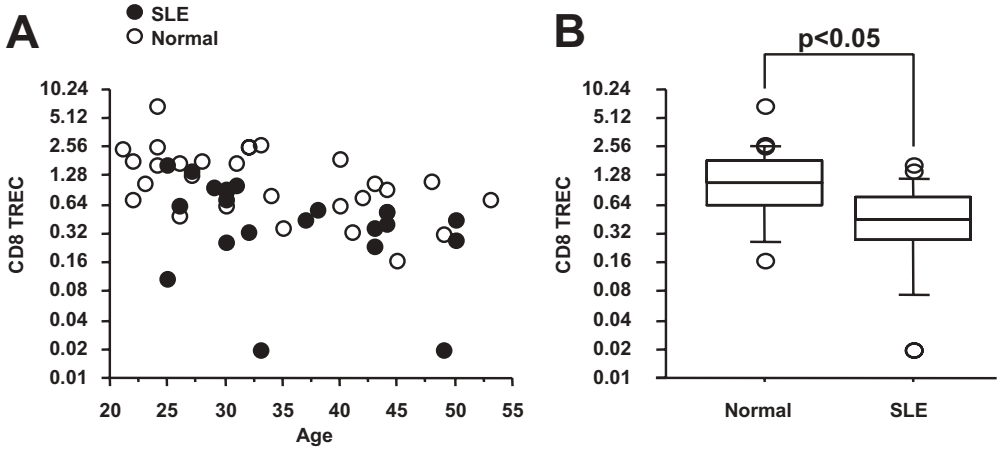

Fig. 2. Signal-joint TREC levels of CD8+ cells in healthy individuals and SLE patients. **A** Age distribution of Sj TREC levels of CD8+ cells. **B** Sj TREC levels of CD8+ cells in healthy individuals and SLE patients. Sj TREC levels are expressed on a log2 scale

20 µl of the supernatant, 5 µl of 10× TRAP Reaction Mix, 1 µl of 50× dNTP mixture, 21.6 µl of distilled water, and 0.4 µl of Taq polymerase were added. The mixture was incubated at 30°C for 10 min, and subjected to PCR using the thermal cycler GeneAMP 9700 (Applied Biosystems, Foster City, CA, USA). The PCR conditions were 30 cycles of 94°C for 30 s and 60°C for 30 s. PCR products were electrophoresed on 12% polyacrylamide gels, stained with SYBER Gold (Molecular Probes Eugene, OR, USA), and photographed with a CCD camera and a UV transilluminator (TOYOBO, Tokyo, Japan). The images obtained were analyzed using NIH image analysis software (National Institute of Health, Bethesda, MD, USA). In this analysis, a ladder of bands larger than 50 bp was regarded as TRAP products, and the total of the TRAP products divided by that of the control was defined as the telomerase activity level.

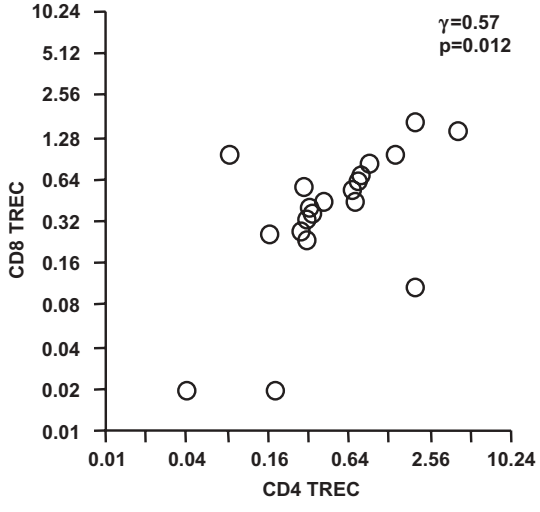

Fig. 3. Comparison of Sj TREC levels between CD4+ and CD8+ levels. Sj TREC levels are expressed on a log2 scale

Statistical analysis

The SLE patients and healthy subject groups were compared by the Mann–Whitney *U* test. The correlations between clinical laboratory data and Sj TREC levels were analyzed using Spearman's rank correlation test. *P* values less than 0.05 were considered to be significant in all analyses.

Results

T cell receptor excision circles' levels are known to decrease with age. Therefore, in this study, we compared Sj TREC levels between SLE patients and age-matched healthy subjects (Figs. 1A, 2A). The mean Sj TREC levels of CD4+ and CD8+ cells in the healthy subject group were 1.22 ± 0.90 and 1.42 ± 0.28, whereas those in the SLE patient group were 0.80 ± 0.92 and 0.57 ± 0.44, respectively. Comparison of the healthy and SLE groups concerning the Sj TREC level of CD4+ cells by the Mann–Whitney *U* test showed a *P* value of 0.035 (Fig. 1B). Similar comparison of both groups concerning the Sj TREC level of CD8+ cells showed a *P* value of 0.001 (Fig. 2B). Thus, the Sj TREC levels of CD4+ and CD8+ cells were significantly lower in the SLE than in the healthy group.

We then analyzed the correlation between the Sj TREC levels of CD4+ and CD8+ cells in the SLE group by Spearman's rank correlation test, and found a positive correlation between them, at a correlation coefficient of 0.57 and a *P* value of 0.012 (Fig. 3). Similar analysis of the correlation between telomerase activity values and Sj TREC levels revealed no significant correlation: the correlation coefficient and *P* value in the CD4+ cells were 0.18 and 0.500, respectively (Fig. 4A), and those in the CD8+ cells were 0.08 and 0.770, respectively (Fig. 4B). The correlations between clinical laboratory parameters and Sj TREC levels were analyzed by Spearman's rank correlation test. The Sj TREC

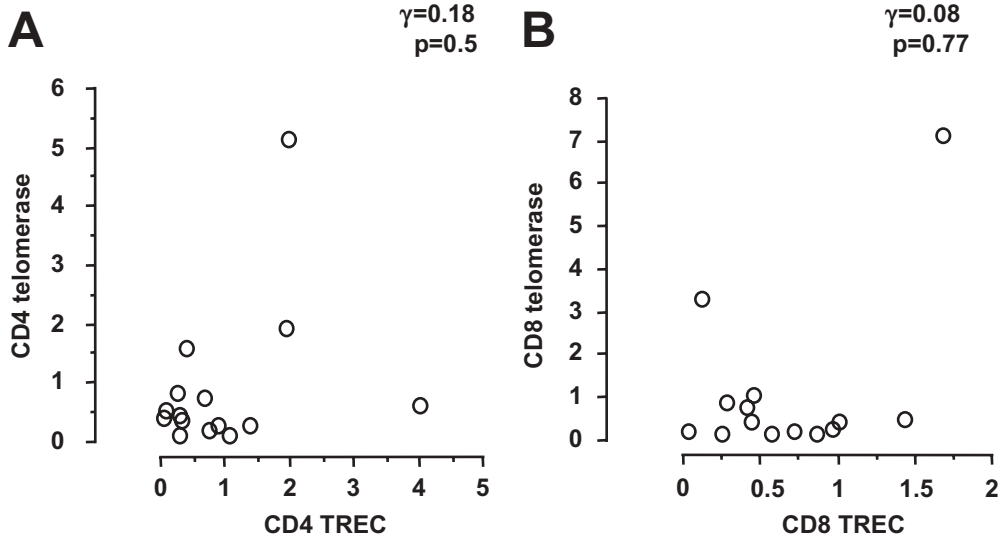

Fig. 4. Comparison of Sj TREC levels and telomerase activity. **A** Comparison of Sj TREC levels and telomerase activity in CD4+ cells. **B** Comparison of Sj TREC levels and telomerase activity in CD8+ cells. Sj TREC levels are expressed on a log2 scale

Table 1. Correlations of peripheral CD4+ cells or CD8+ cells T cell receptor excision circles with various clinical data

	CD 4		CD 8	
	Coefficient	*P*	Coefficient	*P*
WBC	–0.426	0.057	–0.092	0.688
Lymphocyte	–0.107	0.634	0.183	0.425
Hb	–0.019	0.933	0.14	0.541
Thrombocyte	–0.37	0.098	–0.138	0.546
CRP	0.018	0.935	0.199	0.386
C3	–0.381	0.097	0.04	0.867
C4	–0.606	0.010*	–0.198	0.415
CH50	–0.436	0.064	0.011	0.963
IgG	0.664	0.006**	0.467	0.062
IgA	0.232	0.338	0.022	0.93
IgM	0.269	0.267	0.228	0.361
Anti-ds DNA ab	0.368	0.109	0.072	0.761
SLEDAI	0.317	0.157	–0.083	0.718
Prednisolone	–0.272	0.224	–0.083	0.717
Telomerase	0.182	0.496	0.081	0.769

WBC, white blood cell counts (per microliter); lymphocyte, lymphocyte cell counts (per microliter); Hb, hemoglobin (g/dl); Thrombocyte, thrombocyte cell counts (per microliter); CRP, C-reactive protein (mg/dl); C3, complement 3 (mg/dl); C4, complement 4 (mg/dl); CH50, complement activity (U/ml); IgG, immunoglobulin G (mg, dl); IgA, immunoglobulin A (mg, dl); IgM, immunoglobulin M (mg, dl); anti-dsDNA ab, anti-double-stranded DNA antibody (U/ml); SLEDAI, systemic lupus erythematosus disease activity index; Prednisolone, dosage of prednisolone (mg/day); Telomerase, telomerase activity
Coefficient of correlation (*P* value): Spearman's correlation coefficient by rank; * $P < 0.05$; ** $P < 0.01$

levels of CD4+ cells were correlated positively with IgG levels, and negatively with C4 values. No other significant correlations were observed (Table 1).

Discussion

Recently, Kayser et al.[3] reported that SLE patients show lower PBMC TREC levels than healthy individuals. However, the percentage of T cells in the PBMC fraction may be reduced in SLE patients. Therefore, their results may simply have reflected a reduction in T cells in the PBMC fraction. Thus, in this study, we classified T cells into CD4+ and CD8+ fractions to analyze Sj TREC levels, and found that the Sj TREC levels of the peripheral blood CD4+ and CD8+ cells were lower in SLE patients than in the healthy subjects, with a correlation between those of both types of cells in the SLE patients. This suggested that the mechanism causing abnormalities in Sj TREC levels was operative in a stage common to both, that is, at the thymus level. Several studies in murine SLE models have reported on abnormalities of the thymus, leading to the hypothesis of a possible involvement of this organ in the disease pathogenesis.[8–11] Thymus atrophy and a series of abnormalities in peripheral T cell function have also been reported in SLE patients.[12]

However, a decrease in Sj TREC levels also occurs because of causes other than a decreased thymic output. Decreased levels of Sj TREC levels are known to result from increased peripheral T cell division.[1–2] Kayser et al.[3] considered that abnormal TREC levels could result from increased peripheral T cell division.

Telomerase activity is known as an indicator of increased lymphocyte division.[4] Peripheral T cells of patients with SLE are abnormally activated.[13–18] Rapid division of cells is associated with increased telomerase activity.[19–22] We previously reported that the peripheral blood lymphocytes in SLE patients had a high telomerase activity, suggesting the activation of peripheral blood lymphocytes in SLE patients, resulting in rapid cell division.[6] Klapper et al.[23] also reported that peripheral blood CD4+ and CD8+ cells of SLE patients exhibited a high telomerase activity. Thus, to examine whether Sj TREC levels were influenced by increased cell division, we analyzed the correlation between data on Sj TREC levels and telomerase activity values. However, no correlation was noted between Sj TREC levels and telomerase activity values in the CD4+ or CD8+ cells.

Because telomerase activity varies dynamically,[5] the use of its activity level as an indicator of cell division influencing Sj TREC levels remains problematical. However, abnormal Sj TREC levels in the SLE patients were not correlated with telomerase activity levels, suggesting that the decreased Sj TREC levels in the SLE patients cannot be explained by increased cell division alone.

Other factors influencing the Sj TREC level include peripheral T cell death,[2] which has been suggested to occur in SLE patients.[24] Therefore, the relationship between T cell death and Sj TREC levels needs to be investigated.

Although the relationships between Sj TREC levels and clinical laboratory parameters were analyzed, no correlations were found except those between Sj TREC levels and IgG or C4 levels. Similarly, Kayser et al. reported no definite correlations between the Sj TREC level of the PBMC fraction and clinical laboratory parameters. Therefore, much of the clinical significance of the decreased levels of Sj TRECs in CD4+ and CD8+ cells observed in the SLE patients remains unknown.

HIV infection has been reported to be associated with decreased Sj TREC levels.[1,25] The abnormalities observed in the SLE patients in this study have much in common with those reported in HIV-infected patients. It has been shown that HIV patients have also reduced levels of Sj TRECs in CD4+ and CD8+ cells. Ye et al.[25] speculated that, in HIV-infected patients, the reduction in the level of Sj TRECs in CD4+ cells is mainly because of hypofunction of the thymus, and that in CD8+ cells it is mainly because of decreased thymic output and increased peripheral cell division. It has been reported that some HIV-infected patients present with SLE-like symptoms.[26–28] Because the possible presence of immune abnormalities common to both conditions has been noted, it is interesting that reduced levels of Sj TRECs in CD4+ and CD8+ cells were observed in both SLE and HIV-infected patients.

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