

Abnormal Telomerase Activity and Telomere Length in T and B Cells from Patients with Systemic Lupus Erythematosus

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ABSTRACT. Objective. To evaluate the clinical significance of telomerase activity and telomere length in T and B lymphocytes from patients with systemic lupus erythematosus (SLE).

Methods. CD3+ (T cell) and CD19+ (B cell) lymphocytes were isolated from the peripheral blood of SLE patients and healthy controls by means of magnetic bead-coupled antibodies. SLE patients were classified as active or inactive cases according to the SLE Disease Activity Index (SLEDAI). Telomere activity of lymphocytes was measured by telomeric-repeat amplification protocol. Telomere length was measured by flow cytometry-fluorescence *in situ* hybridization.

Results. T cell telomerase activity was significantly higher in patients with both active and inactive SLE than in controls, but was lower than B cell telomerase activity in patients with active SLE, and was not correlated with SLEDAI results. B cell telomerase activity was only significantly higher than in controls in patients with active SLE, and was strongly correlated with SLEDAI. Four laboratory results, anti-dsDNA antibody titer, IgG level, C3 level, and CH50 level, were correlated with B cell telomerase activity. Telomere length in T cells was significantly shorter than in controls. In contrast, the telomere length in B cells did not differ significantly from controls.

Conclusion. In patients with SLE, many T cells divide continuously. Their telomerase activity was higher than that in control T cells, but not so high as to prevent telomere shortening. In contrast, B cells do not divide abnormally in the inactive phase of SLE, but divide rapidly in the active phase. (J Rheumatol 2006;33:1102-7)

Key Indexing Terms:

TELOMERASE ACTIVITY
T LYMPHOCYTE

TELOMERE LENGTH

SYSTEMIC LUPUS ERYTHEMATOSUS
B LYMPHOCYTE

The telomere is a DNA protein complex that is essential for the stabilization of chromosomes¹. The telomere DNA, located at the end of chromosomes, is not replicated by the usual DNA polymerase, and shortens at each cell division, unless replicated^{1,2}. Telomerase is a reverse transcriptase that adds telomere repeat sequences to the end of chromosomes, and is considered to resist telomere shortening so as to maintain telomere structure². In cancer cells, telomerase activation occurs, resulting in the maintenance of telomere length despite cell division, thereby allowing them to divide indefinitely³.

Telomerase activation also occurs in non-diseased cells including lymphocytes⁴. Lymphocytes are activated by some stimuli, and when they are actively dividing, telomerase activity increases^{4,12}. Several studies have investigated telomerase activity in autoimmune diseases¹³⁻¹⁵.

We have reported that telomerase activity in peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE) was strongly correlated with disease activity, and speculated that the abnormal telomerase activity in PBMC was probably due to that in the lymphocytes in PBMC^{16,17}. Later, Klapper, *et al* described abnormal telomerase activity in peripheral blood T and B lymphocytes¹⁸. In our study, we examined telomerase activity and telomere length in peripheral blood T and B cells from patients with SLE, and found interesting correlations between telomerase activity and telomere length and clinical findings.

MATERIALS AND METHODS

Patients. Informed consent was obtained from 34 SLE patients and 17 healthy control subjects, and 20 ml of peripheral blood was collected from each. All SLE patients met the classification criteria of the American Rheumatism Association for SLE. We examined peripheral blood T and B cell telomerase activity and telomere length in 34 and 17 SLE patients, respectively, and in 17 controls. The study was conducted in accord with the Helsinki Declaration.

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Supported by grants from Bio-Venture Research Fund Aid, Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Accepted for publication January 13, 2006.

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The 34 SLE patients consisted of 31 women and 3 men ranging in age from 18 to 64 years (mean 39.15 ± 12.47 yrs). These 34 patients were classified according to the SLE Disease Activity Index (SLEDAI) as having active (SLEDAI ≥ 6) or inactive disease (SLEDAI < 6). Of these 34 patients, 18 had active disease (mean age 40.0 ± 13.19 yrs) and 16 inactive disease (mean age 38.19 ± 11.97 yrs). The 17 patients who were measured for telomere length were all women, ranging in age from 26 to 57 years (mean 39.74 ± 10.57 yrs). The 17 controls, 16 women and one man, ranged in age from 18 to 57 years (mean 37.77 ± 12.56 yrs).

Isolation and purification of T and B cells. A mononuclear cell fraction was isolated from heparinized peripheral blood by Ficoll density gradient centrifugation, and purified into CD3-positive cells (T cells) and CD19-positive cells (B cells) with a magnetic bead cell-sorting system (Miltenyl Biotec, Gladbach, Germany). Finally, flow cytometry with a FACSCalibur (Becton Dickinson, San Jose, CA, USA) showed that purification rates of the T and B cells were $> 95\%$.

Measurement of telomerase activity. Telomerase activity in T and B lymphocytes was measured by telomeric-repeat amplification protocol (TRAP) assay with a TRAPeze telomerase detection kit (Intergen, Purchase, NY, USA) according to a slight modification of the manufacturer's experimental protocol. Each sample of cells (1.0×10^5) was suspended and lysed in $100 \mu\text{l}$ of $1 \times \text{CHAP}$ lysis buffer, homogenized, and incubated on ice for 30 min. The cell lysate was centrifuged at 15,000 RPM for 20 min at 4°C . Twenty microliters of the supernatant, $5 \mu\text{l}$ of $10 \times \text{TRAP}$ buffer, $1 \mu\text{l}$ of $50 \times \text{dNTPs}$, $1 \mu\text{l}$ of TS primer, $1 \mu\text{l}$ of primer mixture, $21.6 \mu\text{l}$ of distilled water, and $0.4 \mu\text{l}$ of Taq polymerase were mixed. The mixture was incubated at 30°C for 10 min, and subjected to polymerase chain reaction (PCR) in a thermal cycler (GeneAmp 9700; Applied Biosystems, Foster City, CA, USA) for 30 cycles of 30 s at 94°C and 30 s at 60°C . The PCR product was electrophoresed on a 12% polyacrylamide gel, which was stained with SYBR Gold (Molecular Probes, Eugene, OR, USA), and photographed with a UV transilluminator equipped with a CCD camera (Toyobo, Tokyo, Japan). Images were analyzed with specific software (National Institutes of Health, Bethesda, MD, USA). All bands > 50 bp were measured with a densitometer, and the sum total of absorbance values was regarded as the amount of TRAP products. The internal control band was also measured by densitometer, and the absorbance was used as the internal control value. Telomerase activity value was defined as the value for the TRAP product divided by the internal control value. All experiments were performed in triplicate, and the mean value was used for analysis.

Measurement of telomere length in lymphocytes by flow cytometry-fluorescence in situ hybridization (FISH). Telomere length in each lymphocyte fraction was measured by flow-FISH¹⁹ according to instructions for the Telomere PNA Kit (Dako Cytomation, Glostrup, Denmark). Purified 1×10^6 lymphocytes from each fraction were mixed well with $300 \mu\text{l}$ of fluorescein isothiocyanate (FITC)-conjugated telomere peptide nucleic acid (PNA) probe (Dako Cytomation) and incubated at 82°C for 10 min and at room temperature overnight in the dark. The lymphocytes were washed twice with wash solution (Dako Cytomation), and stained with $500 \mu\text{l}$ propidium iodide-containing DNA staining solution (Dako Cytomation) at 4°C for 3 h. At least 10,000 cells per sample were measured with a FACSCalibur flow cytometer (Becton Dickinson) in channels F1 and F3. After removal of dividing cells and dead cells by DNA staining and gating, the intensity of FITC fluorescence of the sample cells was measured, and the mean was calculated. To maintain constant sensitivity of the FACSCalibur, standard cells were prepared and measured for fluorescence intensity as described above, and the fluorescence sensitivity of the FACSCalibur was appropriately adjusted. Telomere length in sample cells was expressed as the relative telomere length, defined as the ratio of the mean fluorescence intensity of sample cells to that of standard cells. The human T cell 1301 leukemia cell line (European Collection of Cell Cultures, Wiltshire, UK) was used as a standard cell, according to instructions from Dako Cytomation (Figure 1).

Analysis of results. The Kruskal-Wallis test was used to detect differences among the active SLE group, the inactive SLE group, and the controls. The Mann-Whitney U test was used post-hoc to test differences between pairs of

groups, and Bonferroni-corrected p values < 0.0167 ($= 0.05/3$) were considered significant. Correlation between SLEDAI or clinical data and telomerase activity was analyzed by Spearman's rank correlation test. Differences in telomere length between control and SLE groups were analyzed by Mann-Whitney U test.

RESULTS

Peripheral blood T cell telomerase activity levels were compared among the active SLE, inactive SLE, and control groups (Figure 2). The Kruskal-Wallis test showed significant differences among the 3 groups. Next, comparisons between pairs of groups were performed by the Mann-Whitney U test. For telomerase activity, the active SLE and control groups were significantly different ($p < 0.01$), as were the inactive SLE and control groups ($p = 0.004$). In contrast, the active SLE group did not differ significantly from the inactive SLE group ($p = 0.162$). Thus, T cell telomerase activity in the active and inactive phases of SLE was higher than normal, but the telomerase activity in the active phase did not differ from that in the inactive phase. Similarly, the Kruskal-Wallis test revealed significant differences in peripheral blood B cell telomerase activity among the 3 groups (Figure 2). The Mann-Whitney U test showed that p values between the active SLE and control groups and between the active and inactive SLE groups were less than 0.001, but between the inactive SLE and control groups the p value was 0.505. Thus, B cell telomerase activity was increased only in the active phase of SLE.

The correlation between T cell telomerase activity and SLEDAI results was analyzed by Spearman's rank correlation test (Figure 3). The p value between T cell telomerase activity and SLEDAI was 0.312, showing no correlation. Similar analysis revealed a strong positive correlation between B cell telomerase activity and SLEDAI ($p < 0.01$; Figure 3). To examine the relationship between B cell telomerase activity and SLEDAI, between anti-dsDNA antibody titer and SLEDAI, and between complement titer and SLEDAI, we used a modified SLEDAI (M-SLEDAI) from which the items for anti-dsDNA antibody titer and complement titer were excluded¹⁷. We examined the relationships between the M-SLEDAI and B cell telomerase activity, between M-SLEDAI and anti-dsDNA antibody titer, and between M-SLEDAI and complement titer (CH50): we found positive correlations between M-SLEDAI and B cell telomerase activity ($r = 0.632$) and between M-SLEDAI and anti-dsDNA antibody titer ($r = 0.451$), and a negative correlation between M-SLEDAI and CH50 ($r = -0.425$). Thus, B cell telomerase activity was most strongly correlated with M-SLEDAI.

The relationship between peripheral B cell telomerase activity and each laboratory test item was analyzed by Spearman's rank correlation test (Table 1). B cell telomerase activity was positively correlated with IgG level ($p = 0.001$) and anti-dsDNA antibody titer ($p = 0.002$), and was negatively correlated with C3 level ($p = 0.03$) and CH50 ($p = 0.003$). No significant correlation was found between peripheral

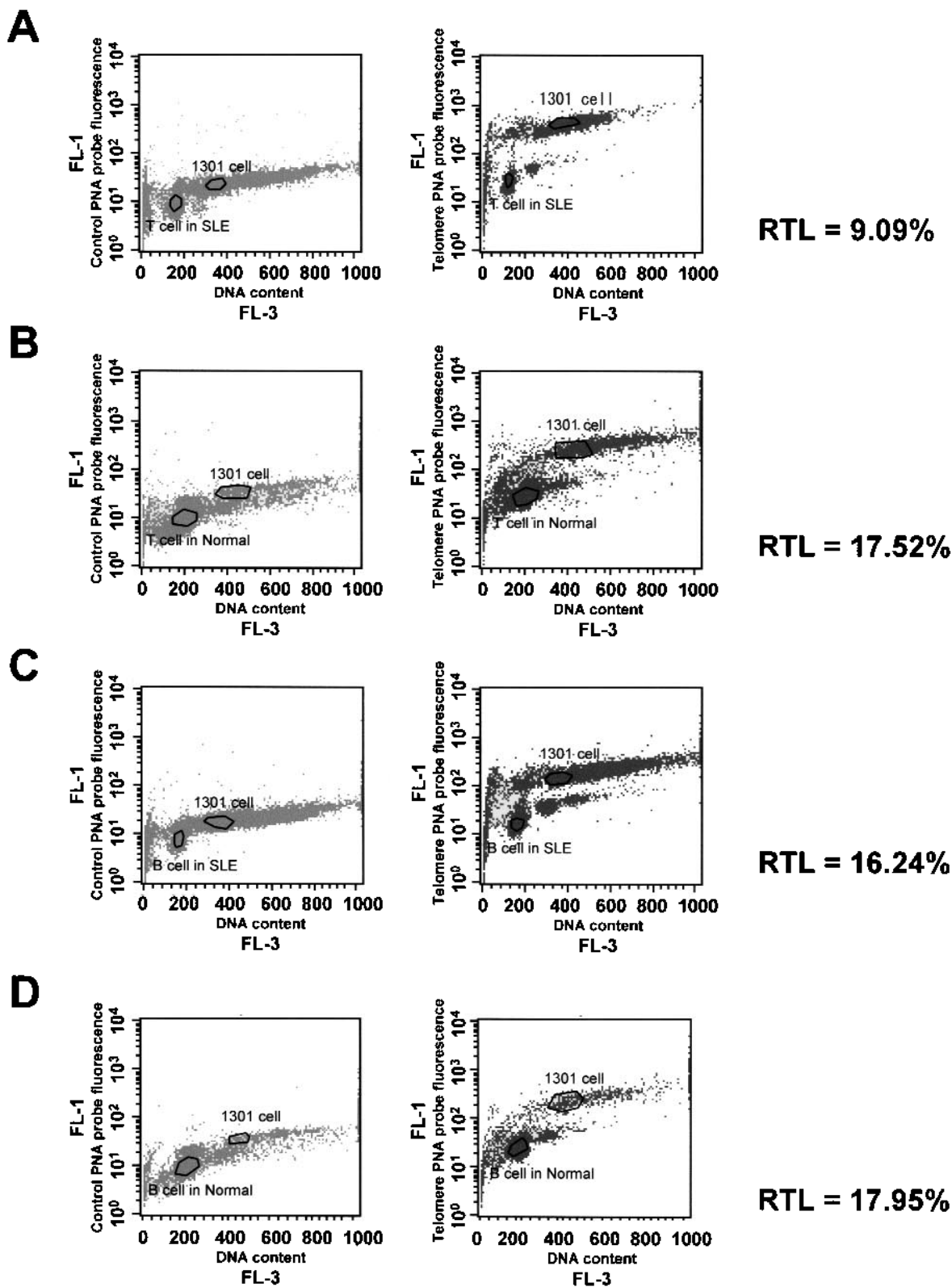


Figure 1. Analysis of telomere length by flow-FISH. FL-1 and FL-3 represent FITC and PI channels, respectively. Fluorescence of samples hybridizing with the FITC-telomere sequence probe is detected in FL-1. Since 1301 cells are tetraploid, they are separated in FL-3 from the diploid sample cells. Results using the non-telomere sequence control probe represent the background (autofluorescence). Analysis data for representative samples are shown. A. Telomere length of T cells from a 30-year-old woman with SLE. Relative telomere length (RTL) = 9.09%. B. Telomere length of T cells from a healthy 27-year-old female control. RTL = 17.52%. C. Telomere length of B cells from a 30-year-old woman with SLE. RTL = 16.24%. D. Telomere length of B cells from a healthy 27-year-old female control. RTL = 17.95%. PNA: peptide nucleic acid.

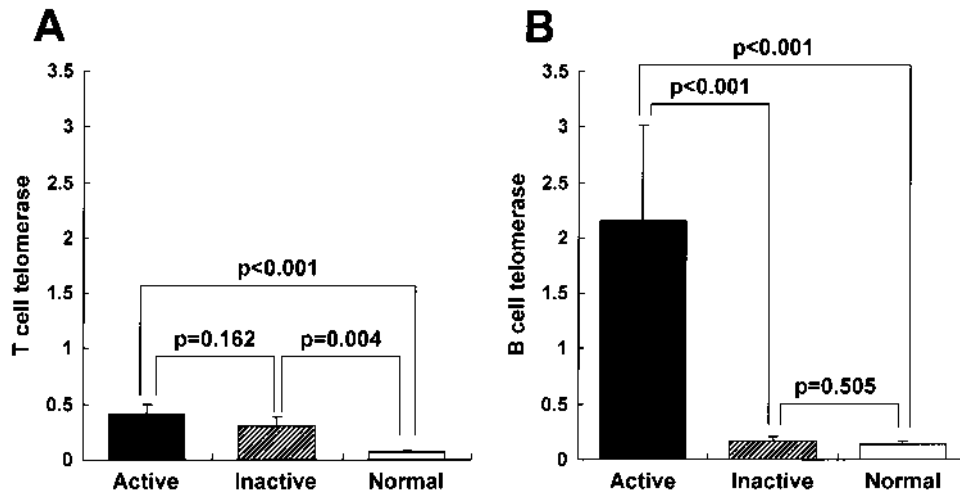


Figure 2. Telomerase activity and telomere length in T and B cells from SLE patients, classified as active and inactive SLE according to SLEDAI. Telomerase activity was measured in T and B cells and compared with control T and B cells. A. Mean T cell telomerase activity (standard error) was higher in active and inactive SLE than in controls. No difference in telomerase activity was noted between active and inactive SLE groups. B. Mean B cell telomerase activity (SE) was higher in active SLE than in controls; there was no difference in telomerase activity between the inactive group and controls.

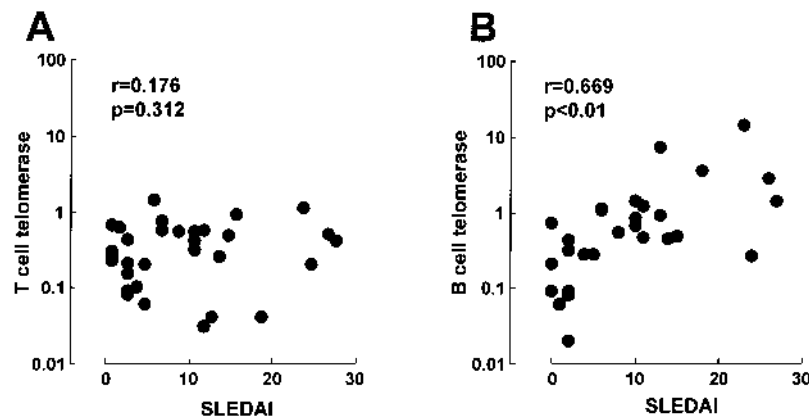


Figure 3. Relationship between peripheral T or B cell telomerase activity and SLEDAI. A. No correlation between T cell telomerase activity and SLEDAI. B. A correlation was found between B cell telomerase activity and SLEDAI.

blood T cell telomerase activity and each laboratory test item (data not shown).

The Mann-Whitney U test showed that p values between the control and SLE groups were 0.0127 (< 0.05) and 0.3062 (> 0.05) for peripheral T cell and B cell telomere lengths, respectively (Figure 4). Thus, the T cell telomere length was shorter in the SLE group than in the control group, but there was no intergroup difference in B cell telomere length.

DISCUSSION

Peripheral T cell telomerase activity was similarly higher in patients with active and inactive SLE than in healthy controls. Klapper, *et al* reported that peripheral CD4+ and CD8+ T cell telomerase activity was increased, with no statistical signifi-

cance¹⁸. In contrast, we observed a significant difference between the SLE patients and healthy controls. Since we analyzed CD3+ cells instead of CD4+ and CD8+ cells, our data cannot be directly compared with Klapper, *et al*. However, analysis of CD4+ and CD8+ cells in more patients may yield a significant difference. Interestingly, telomerase activity in peripheral blood B cells derived from patients with active SLE was higher than that in controls and that in T cells; however, telomerase activity in B cells derived from patients with inactive SLE did not differ from that in B cells from controls. Correlation analysis showed T cell telomerase activity was not correlated with SLEDAI, but B cell telomerase activity was.

Next, we examined the degree of correlation between M-SLEDAI and B cell telomerase activity, anti-dsDNA antibody

Table 1. Correlations of peripheral B cells telomerase activity with clinical data.

	y	p	Result
WBC count, / μ l	-0.135	0.439	NS
Lymphocyte cell count, / μ l	-0.298	0.115	NS
Hemoglobin, g/dl	-0.073	0.674	NS
Platelets, / μ l	0.038	0.827	NS
C-reactive protein, mg/dl	0.332	0.056	NS
C3, mg/dl	-0.410	0.030	S
C4, mg/dl	-0.285	0.154	NS
CH50, U/ml	-0.547	0.003	S
IgG, mg/dl	0.654	0.001	S
IgA, mg/dl	0.255	0.194	NS
IgM, mg/dl	0.349	0.075	NS
dsDNA	0.553	0.002	S
Proteinuria	0.171	0.341	NS
Prednisolone, mg/day	-0.218	0.248	NS

WBC: white blood cell count; thrombocyte cell counts. Coefficient of correlation (p value): Spearman's correlation coefficient by rank. S: significant, NS: not significant.

titer, and CH50; we found that B cell telomerase activity was more strongly correlated with M-SLEDAI than with anti-dsDNA antibody titer or CH50. These results suggest that the activity of PBMC-derived telomerase, which was correlated with disease activity in our previous work¹⁷, mainly reflected B cell telomerase activity. The results also suggest that B cells, reported by Klapper, *et al* to have high telomerase activity¹⁸, were mainly derived from patients with active disease.

Next, we examined the relationship between B cell telomerase activity and laboratory data. Previously we reported that PBMC telomerase activity was correlated with M-SLEDAI, but not with C3 level or anti-dsDNA antibody titer, and noted that this correlation should be investigated further¹⁷. In this study, we analyzed B cells instead of PBMC, and found correlations between B cell telomerase activity and C3 level, and between B cell telomerase activity and anti-dsDNA antibody titer. Although we previously reported that PBMC telomerase activity was not correlated with IgG level¹⁷, B cell telomerase activity was correlated with IgG level in this study. In addition, we previously reported that PBMC telomerase activity

was correlated with level of C-reactive protein (CRP) and was negatively correlated with hemoglobin level, and that the meaning of these correlations was not clear¹⁷. However, in the current study we found no correlation between B cell telomerase activity and CRP or hemoglobin levels. C3 level and anti-dsDNA antibody titer are considered to be the most clinically useful indicators of SLE activity. IgG levels are considered to be elevated by the polyclonal activation of B cells²⁰. Therefore, the correlations between these items and B cell telomerase activity suggest that the disease activity of SLE is closely associated with B cell activation.

We also measured telomere length in the T and B cell fractions. Since lymphocyte telomere length changes with age, we carefully selected age-matched controls. Patients with active and inactive SLE might have different telomere lengths; however, our analysis showed that this was not the case (data not shown). In addition, similarly to the results for PBMC analysis¹⁷, no definite correlation was found between telomerase activity and telomere length (data not shown). Therefore, the patients were not divided into those with active or inactive disease for comparison of telomere lengths. The results showed that the peripheral blood T cell telomere length was shorter in the SLE patients than in the controls. In contrast, the peripheral blood B cell telomere length did not differ between the SLE patients and controls.

Based on results of the analysis of telomerase activity and telomere length, we speculate as follows. In patients with SLE, many T cells divide continuously, and their telomerase activity is higher than that in healthy subjects, but not so high as to prevent telomere shortening. Indeed, telomerase activity was lower in T cells than in B cells. On the other hand, SLE patients do not have more B cell divisions than healthy subjects. Even if many B cell divisions occur, increased telomerase activity prevents the shortening of telomeres. Further, in B cells, telomerase activity was elevated in the active disease phase, but not in the inactive phase. These findings suggest that B cells do not undergo more frequent cell divisions in the inactive phase of SLE, and that in the active phase, B cells divide rapidly, but increased telomerase activity prevents telomere shortening.

Many studies have reported that T and B cells are activat-

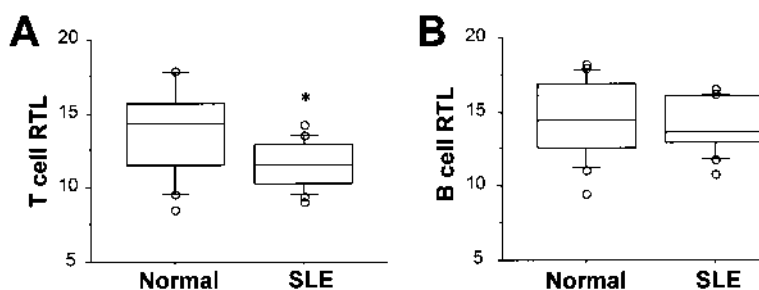


Figure 4. Telomere lengths in peripheral blood T and B cells from SLE patients and controls. A. T cell telomere length from SLE patients was shorter than that in controls. B. B cell telomere lengths did not differ between the SLE and control groups. * $p < 0.05$.

ed in SLE²⁰⁻²⁶; however, few studies have addressed the question by applying the same measurement methods to T and B lymphocytes. In this respect as well, the data we obtained were interesting. The measurements for telomere length and telomerase activity suggest that T cells are continuously activated in patients with SLE, but this alone does not cause the disease to develop, and the disease becomes manifest when B cell activation also occurs. Although it is not clear whether B cell activation is mediated by T cells, the inhibition of B cell activation may at least lead to the suppression of disease activity. Indeed, anti-CD20 antibody has recently been reported to be effective for SLE^{27,28}. However, the ultimate treatment of SLE may require the inhibition of continuous T cell activation.

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