Telomere Lengthening in CD8$^+$ Cells in Polyclonal *In Vitro* Stimulation is Associated with an Increase in Protein Content of Catalytic Subunit of Telomerase (hTERT)

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Culturing of polyclonally activated T lymphocytes for 7 days *in vitro* leads to telomere lengthening in CD8$^+$, but not CD4$^+$ lymphocytes. Under these conditions, CD8$^+$ lymphocytes more intensively express telomerase catalytic subunit protein (hTERT) and divide more often than CD4$^+$ lymphocytes. It changes the ratio of CD4$^+$ and CD8$^+$ subpopulations in favor of the latter by the end of culturing.

Key Words: *T lymphocytes; telomeres; telomerase; hTERT; CFSE*

Antiergotypic response is a mechanism controlling expansion of antigen-activated cells. This is an immune response against activation markers (CD25 and some others united by the notion of ergotop) typically expressed on T cells stimulated via T-cell receptor [10]. Induction of the anti-ergotypic response via immunization with T cells polyclonally activated with antibodies to T-cell receptor (aCD3) is used for the treatment of immunopathological diseases [4,5].

For obtaining sufficient amount of cells for vaccination, they are cultured *in vitro* in the presence of aCD3 and IL-2. During culturing, T cells divide several times, which leads to changes in the ratio of CD4$^+$ and CD8$^+$ cells and expression of surface markers (CD25, CD28, *etc*.) and intracellular molecules including a range of lymphocyte-produced cytokines [1,3,7]. The number of cell divisions can be limited as a result of telomere shortening during each division. Critical telomere shortening inhibits cell proliferation, and with additional stimulation leads to apoptosis [6]. Telomerase activation and expression of mRNA and protein of catalytic subunit (hTERT) associated with lymphocyte stimulation *in vivo* и *in vitro* represent the main mechanism for maintaining telomere length in proliferating cells [3,11]. Temporary elongation of telomeres in proliferating T lymphocytes after stimulation was also shown [9].

Thus, the study of T cells during culturing is important both for the characteristics of cell substrate for T cell vaccine production, and the study of the features of proliferation in T lymphocyte populations.

Here we present the results of measuring telomere length in CD4$^+$ and CD8$^+$ lymphocytes depending on the number of cell divisions and the level of protein production of catalytic subunit of telomerase (hTERT) over 7 days in culture under conditions of polyclonal *in vitro* stimulation.

MATERIALS AND METHODS

Peripheral blood of healthy donors was used. Mononuclear cells were isolated by centrifugation of heparinized venous blood in Ficoll-verografin density gradient. Lymphocytes (2×10^6 cells) were cultured in 1 ml complete medium consisting of RPMI-1640 with 10% FCS (HyClone), 0.3 mg/ml glutamine, 50 μg/ml gentamicin, and 25 μg/ml thienam. The cells were activated with anti-CD3 antibodies (1 μg/ml; MedBioSpektr) in

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the presence of IL-2 (100 U/ml; Biotech). Telomere length was assessed using the Flow-FISH technique as described by us previously [2]. Leukocytes were washed with phosphate buffered saline with 0.1% BSA (PBS-BSA; Sigma), labeled with biotinylated mouse anti-CD4 (CD8) antibodies (Becton Dickinson), incubated for 20 min, washed with PBS/BSA, and then Cy5-streptavidin (Amersham Bioscience) was added for 20 min. After washout, the cells were incubated in 4 mM BS^3 solution (Bis(sulfosuccinimidyl)suberat; Pierce) for 30 min followed by addition of Tris (Sigma) in a final concentration of 20 mM for 15 min and washing. Hybridization was performed in 70% formamide (Sigma) with 20 mM Tris and 1% BSA. A probe $(CCCTAA)_3$-FITC (0.3 μg/ml, Eurogentec Ltd) was used to detect telomeric repeats. The samples were heated at 80°C for 10 min and incubated for 3 h at 25°C. Cells were transferred to flow cytometry tubes and washed twice with 70% formamide and once with PBS-BSA with 0.1% Tween. The precipitate was resuspended in 0.5 ml PBS-BSA with 2 μl/ml 7-AAD (ICN Biomedicals Inc). Analysis was performed using flow cytofluorometer (FACS Calibur, Becton Dickinson) and Cell Quest pro software (Becton Dickinson). The fluorescence signal of the probe reflecting the amount of telomeric DNA was evaluated as average fluorescence intensity of studied cells in G0/G1 phase of the cell cycle minus fluorescence of the samples that were used for FISH without a probe (control). The absolute length of telomeres was calculated by the formula: $Y=2042+28{,}099\times X$, where Y is the mean telomere length in nucleotide pairs and X is probe fluorescence. The equation was deduced by parallel measurements of telomere length using methods of Southern Blotting and Flow-FISH in 5 donors [2].

For measuring hTERT protein content in intact or cultured peripheral blood lymphocytes, mononuclear cells were stained first with monoclonal antibodies of surface markers CD4 and CD8 (Promix) in the amount recommended by the manufacturer as described previously [5], then fixed in 1% paraformaldehyde, permeabilized in 0.2% Tween-20 (Sigma), incubated with rabbit monoclonal antibodies to hTERT (Epitomics; antibody titer was 1:200), washed, and stained with FITC-labeled anti-rabbit secondary antibodies (Abcam). Then the cells were washed twice and resuspended in PBS-EDTA. The samples were analyzed by flow cytofluorometry. The number of cell divisions was determined using vital dye CFSE (Molecular probes). The cells were stained before culturing (2 μM in RPMI-1640 for 15 min) followed by washing with RPMI-1640 with 10% FCS (HyClone) [1].

RESULTS

Individual changes in telomere length of $CD4^+$ and $CD8^+$ cells after 7 days in culture are shown in Fig. 1. The mean length of telomeres in $CD4^+$ lymphocytes did not change significantly (6359±269 b.p. before and 6328±285.5 b.p. after culturing) whereas the mean length of telomeres in $CD8^+$ lymphocytes significantly increased from 5941±216 b.p. to 6320±232.9 b.p. ($p<0.05$; paired Wilcoxon test).

The relative content of hTERT protein increased on day 3 of lymphocyte culturing in the presence of aCD3. In this case, its amount was significantly higher in $CD8^+$ mononuclear cells and in stimulated lymphocytes compared to $CD4^+$ cells (Fig. 2). This probably helps to maintain telomere length in $CD4^+$ lymphocytes and promotes telomere lengthening in $CD8^+$ lymphocytes during stimulation, which agrees with published data demonstrating temporary elongation of lymphocyte telomeres during the first 2 weeks of culturing [9].

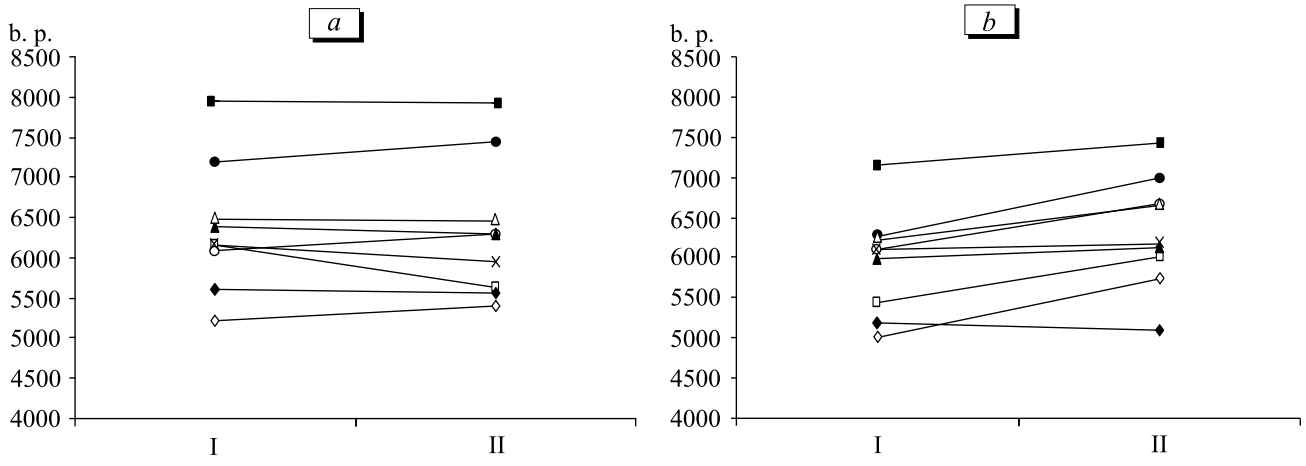

Fig. 1. Telomere length in activated $CD4^+$ (*a*) and $CD8^+$ lymphocytes (*b*) before (I) and after (II) *in vitro* culturing for 7 days ($n=9$).

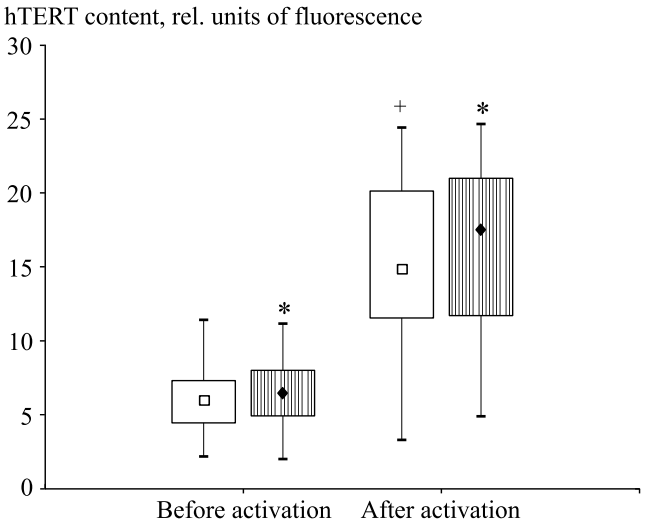

Fig. 2. Changes in the content of telomerase subunit hTERT after activation of lymphocytes cultured *in vitro* (n=20). Open symbols: $CD4^+$; dark symbols: $CD8^+$. *p<0.05 in comparison with $CD4^+$ lymphocytes (paired Wilcoxon test). ^+p<0.05, significance of differences between stimulated and intact lymphocytes in both subpopulations.

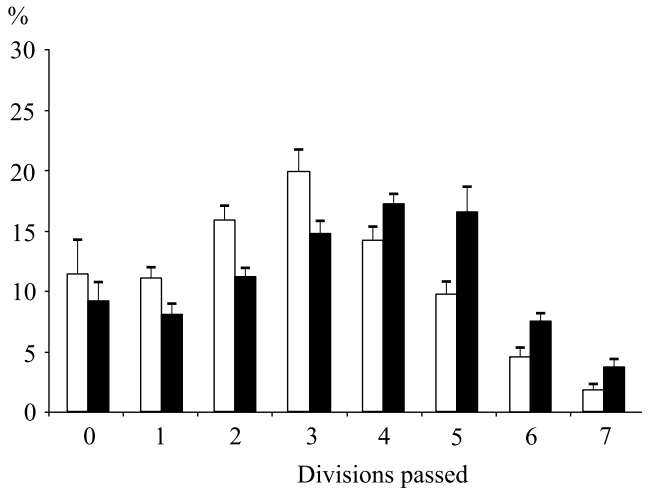

Fig. 3. Percentage of $CD4^+$ (light bars) and $CD8^+$ lymphocytes (dark bars) passing different numbers of cell divisions in vitro over 7 days in culture (n=7). The numbers of $CD4^+$ and $CD8^+$ cells significantly differ for each division, except 0 (p<0.05; paired Wilcoxon test).

Since telomere elongation can only occur during cell division, we determined the number of divisions completed in the culture using vital dye CFSE (Fig. 3). The cells underwent maximum 7 divisions; the majority of $CD4^+$ lymphocytes passed 3 divisions and the greater part of $CD8^+$ lymphocytes passed 4 and 5 divisions. Thus, higher production of protein hTERT corresponds to significant lengthening of telomeres and higher proliferative activity of $CD8^+$ cells. This can determine quantitative changes in the composition of lymphocyte culture: $CD4^+$ and $CD8^+$ lymphocytes before culturing constituted 65.5±2.64 and 34.5±2.64%, respectively, and after culturing 47.9±3.25 and 52.1±3.25% (changes are significant for both populations, p<0.05; paired Wilcoxon test).

These findings suggest that polyclonal activation of T lymphocytes *in vitro* provides telomere lengthening only in $CD8^+$ lymphocytes possibly due to higher activation of telomerase and proliferative activity compared with $CD4^+$ lymphocytes, which must be taken into account when preparing T-cell vaccines.

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