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J Immunol (2000) 165 (8): 4742–4747.

<https://doi.org/10.4049/jimmunol.165.8.4742>

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Telomerase Activity Is Increased and Telomere Length Shortened in T Cells from Blood of Patients with Atopic Dermatitis and Psoriasis¹

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We studied telomerase activity and telomere length in PBMC and purified CD4⁺ and CD8⁺ T cells from blood obtained from a total of 32 patients with atopic dermatitis, 16 patients with psoriasis, and 30 normal controls. The telomerase activity was significantly increased in PBMC from the patients compared with PBMC from normal donors. This increase was most pronounced in the subpopulation of CD4⁺ T cells, which were significantly above the activity of the CD8⁺ T cells in atopic dermatitis, psoriasis patients, and control persons. The telomere length was significantly reduced in all T cell subsets from both atopic dermatitis and psoriasis patients compared with normal individuals. Furthermore, the telomere length was found to be significantly shorter in CD4⁺ memory T cells compared with the CD4⁺ naive T cells, and both of the cell subsets from diseases were shown to be significantly shorter telomere length than the same cell subsets from normal controls. No significant difference was observed between CD8⁺CD28⁻ and CD8⁺CD28⁺ T cell populations in both diseases. However, the telomere length of CD8⁺CD28⁺ T cells from both diseases was significantly shorter than CD8⁺CD28⁺ T cell subsets from normal donors. In conclusion, the increased telomerase activity and shortened telomere length indicates that T lymphocytes in atopic dermatitis and psoriasis are chronically stimulated and have an increased cellular turnover in vivo. *The Journal of Immunology*, 2000, 165: 4742–4747.

Telomeres are specialized nucleoprotein structures that act as protective caps at the end of each chromosome (1). Telomere length reflects the proliferative potential of the cell (2, 3) as they shorten after each cell division (4, 5). The gradual decrease in telomere length is believed to be a biological clock, which leads to cellular senescence and growth arrest, when critically short telomeres are reached (6–8). The enzyme telomerase stabilizes telomere length by adding new blocks of TTAGGG during cell division, thus preventing shortening of telomeres and cellular senescence (9–11). The concept at the moment is that germ-line cells or immature cells have increased telomerase activity and long telomeres, whereas somatic cells have low or no telomerase activity and normal telomere length. Malignant cells have increased telomerase activity and short telomere length (12, 13).

Previously, it was considered that somatic cells do not express telomerase activity, whereas this activity could be readily demonstrated in reproductive cells and tumor cells (14). However, recent reports have demonstrated the presence of telomerase activity in peripheral blood leukocytes and activated lymphocytes (15–18), hair follicles (19), and intestinal mucosal cells (20).

Because telomere length and telomerase activity have mostly been studied in malignant diseases (14), there is little information regarding the chronic inflammatory skin diseases such as atopic dermatitis (AD)³ and psoriasis, which are not associated with internal malignancy. Previously we have reported that telomerase activity is spontaneously increased in blood lymphocytes from AD patients and correlates with cellular proliferation (21). In the present study we demonstrate that there is a significant increase in telomerase activity of CD4⁺ and CD8⁺ T cells from peripheral blood together with a reduced telomere length of lymphocytes, indicating that a major portion of the lymphocytes have gone through a high number of cell divisions in vivo.

Materials and Methods

Patients and healthy persons

The control group consisted of 30 healthy blood donors (15 males, 15 females; age range 20–84 years; mean age 41 years). The AD group consisted of 32 patients (17 males, 15 females; age range 17–59 years; mean age 37 years) with moderate to severe disease activity. Mean IgE level was 6176 U/ml. The diagnosis was established according to the criteria of Hanifin and Rajka (22). Among the patients with AD, we studied PBMC obtained from 32 persons. PBMC and CD4⁺ and CD8⁺ T cells were investigated in seven patients, and PBMC and CD4⁺ naive (CD45RA⁻), CD4⁺ memory (CD45RO⁺), CD8⁺CD28⁺, and CD8⁺CD28⁻ T cells were further studied for telomere length assay in eight patients.

The psoriasis group consisted of 16 patients (11 males and 5 females; age range 20–84 years; mean age 47 years). PBMC and CD4⁺ and CD8⁺ T cells were studied in six patients, and PBMC and CD4⁺CD45RA⁺, CD4⁺CD45RO⁺, CD8⁺CD28⁺, and CD8⁺CD28⁻ cells were studied for telomere length assay in six patients.

None of the patients received systemic treatment before the collection of blood. Patients and healthy controls were informed according to the Helsinki Declaration and gave their consent. The Ethical Committee of Aarhus County approved the investigation.

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Received for publication April 17, 2000. Accepted for publication July 26, 2000.

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¹ K.W. is supported by grants from the NOVO-NORDIC Center for Studies of Growth at the University of Aarhus and the from Aage Bangs Foundation. N.H. is supported by the LEO Pharmaceutical Research Foundation. This work was supported by the NOVO-NORDISK Foundation, the Velux Foundation, the University of Aarhus, the Institute of Experimental and Clinical Research at Aarhus University Hospital, the Danish Asthma and Allergy Association, and the Danish Psoriasis Association.

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³ Abbreviations used in this paper: AD, atopic dermatitis; CI, confidence intervals; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment.

Preparation of PBMC and purified CD4⁺ and CD8⁺ T cells

PBMC were isolated from heparinized blood using Lymphoprep (Nycomed Pharm AS Diagnostics, Oslo, Norway) density gradient centrifugation. CD4⁺ and CD8⁺ T cells were further separated by positive selection using Dynal beads (Dynal, Oslo, Norway) according to the manufacturer's instructions. The purity of the CD4⁺ and CD8⁺ T cell populations was >96% by FACS analysis.

We also isolated subsets of CD4⁺ naive and memory T cells and CD8⁺CD28⁺ and CD8⁺CD28⁻ T cell subsets from peripheral blood to determine their telomere length. Briefly, positive selected CD4⁺ T cells were incubated for 30 min on ice with an anti-CD45RO (UCHL-1) mAb (Immunotech, Marseille, France) to remove memory cells for isolation of naive T cells (CD4⁺CD45RA⁺/CD45RO⁻) and with an anti-CD45RA (ALB11) mAb (Immunotech) for isolating memory (CD4⁺CD45RA⁻/CD45RO⁺) T cells. Cells that bound Ab were removed by anti-mouse IgG-coupled magnetic beads (Dynal), and the remaining cells were collected and subjected to flow cytometry and telomere analysis. The CD8⁺CD28⁺ and CD8⁺CD28⁻ T cell subsets were separated according to the method described by Monteiro et al. (23). In brief, CD4⁺ T cells were depleted by two rounds of CD4⁺ depletion from PBMC using CD4⁺ Dynal beads. The CD4-depleted PBMC (<1% CD4⁺ T cells) were then subjected to CD28⁺ cell isolation. The CD28⁺ cell separation was performed using a CD28 mAb (clone CD28.1, IgG1 isotype, DAKO, Glostrup, Denmark) followed by incubation with pan-mouse IgG magnetic beads (Dynal). The cells bound to the beads were designated CD8⁺CD28⁺. The remaining unbound cells were <1% positive for CD28 by FACS analysis on all samples, and they were then positively selected with CD8⁺ Dynal beads yielding a CD8⁺CD28⁻ T cell population.

Flow cytometry analysis

CD4⁺-FITC, CD8⁺-FITC, CD28⁺-FITC, CD45RO⁺-FITC, and CD45RA⁺-RPE were bought from DAKO. Approximately 1 million cells were stained with FITC- or RPE-conjugated mAb and analyzed with FACScan (Coulter) according to the manufacturer's instructions.

Telomerase assay

Telomerase activity was measured using telomerase PCR ELISA (Roche-Molecular Biochemicals, Basel, Switzerland) based on the telomeric repeat amplification protocol (TRAP) method described by Kim et al. (11). Two million cells were taken to an Eppendorf tube and pelleted at 3000 × *g* for 10 min in a refrigerated centrifuge at 4°C. Then, 200 μl cold lysis reagent was added and incubated on ice for 30 min and centrifuged at 15,000 × *g* for 20 min at 4°C. Supernatant was collected, and the protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA) using BSA as control. Alkaline phosphatase was assayed (Sigma Diagnostics, St. Louis, MO) to evaluate the quality of the cell extract (24). When performing the TRAP assay, 1 μg of cell protein was analyzed according to the manufacturer's instructions. Briefly, telomeric repeats were added to a biotin-labeled primer during the first reaction. Then, the elongation products were amplified by PCR. An aliquot of PCR product was denatured; hybridized to a digoxigenin-labeled, telomeric repeat-specific probe; and bound to a streptavidin-coated 96-well plate. An Ab to digoxigenin, conjugated to peroxidase, was subsequently added, and binding to digoxigenin was visualized by virtue of the ability of the enzyme to metabolize tetramethyl benzidine so as to produce a colored reaction product. The absorbance of the samples was measured at 450 nm (reference wavelength 620 nm) using an ELISA microtiter reader (Labsystems iEMS, Helsinki, Finland) within 30 min after addition of the stop reagent. Each sample was measured as duplicates. Mean OD values of duplicate measurements were recorded as telomerase activity. The quantitative value of telomerase activity was standardized against the protein concentration. Immortalized human kidney cells (293 cells) were used as positive control. Inactivation of telomerase protein at 85°C for 10 min was used as negative control. The PCR reaction without sample was used as PCR control. All criteria were fulfilled according to the kit requirements (25).

Southern blot analysis of terminal restriction fragment (TRF)

Three million cells were used for the preparation of high molecular weight DNA by the Nucleon BACC2 DNA extraction kit (RPN 8502, Amersham Life Science, Buckinghamshire, U.K.). The mean length of TRF was measured using the TeloQuant telomere length assay kit (PharMingen, San Diego, CA) with a few modifications. Briefly, 2.5 μg of purified DNA was digested with *HinfI/RsaI* mixture (10 U each), separated on 0.6% agarose gel electrophoresis in 1× Tris-acetate-EDTA, and transferred onto a nylon membrane (Hybond N⁺, Amersham Life Science) according to the protocol described by the supplier. After rinsing in 2× SSC and neutralization

solution, the filter was hybridized with a specific biotinylated detection telomere probe overnight at 65°C. The filter was washed twice in 2× SSC-0.1% SDS for 5 min each time at room temperature and then placed in 0.2× SSC-0.1% SDS solution at 37°C for 15 min with one repeat. To determine the TRF length, the hybridized probe was visualized by chemiluminescent detection, which detected TRF DNA on Hyperfilm (Amersham Life Science). Mean TRF length was defined as $\sum(OD_i \cdot L_i) / \sum(OD_i)$, where OD_i is the signal intensity and L_i is the length of the DNA at position i . The amount of telomeric DNA was calculated by integrating the volume of each smear using ImageQuant software (Molecular Dynamics).

Statistical analysis

Unless otherwise stated, the data in the text and figures are expressed as mean ± SD. SPSS software (version 10.0, Chicago, IL) was used to perform unpaired comparison with the two-tailed Student *t* test for testing the significance of values between groups. To make sure that age is independent of the effects of diseases, we performed linear regression analysis including age as a continuous variable as well as a categorical variable by dividing age at telomere length measured in four age groups. Significance was accepted for *p* values <0.05 and 95% confidence intervals (CI).

Results

Telomerase activity in T lymphocytes from patients with AD and psoriasis is increased compared with normal controls

To demonstrate whether the T cell was activated in peripheral blood, we assayed the telomerase activity in lymphocytes from both AD and psoriasis. Fig. 1 shows the telomerase activity in PBMC and CD4⁺ and CD8⁺ T cells from healthy controls and from AD and psoriasis patients. An increased telomerase activity was found in PBMC from patients with AD and psoriasis compared with freshly isolated PBMC from healthy donors. Looking at CD4⁺ and CD8⁺ subsets, we were able to show that CD4⁺ T cells from blood of patients with AD and psoriasis expressed higher levels of telomerase activity than those of normal persons (*p* < 0.005). CD8⁺ T cells, on the other hand, expressed significantly lower telomerase activity than the CD4⁺ T cells in both AD and psoriasis (*p* < 0.01), but they still had a higher level of activity than did CD8⁺ T cells from normal donors (*p* < 0.01) (Fig. 1).

Reduced TRF length on T cell subsets

As we found that telomerase activity was increased in PBMC and CD4⁺ and CD8⁺ T cells from patients with AD and psoriasis, we questioned whether the increased telomerase activity could rescue the shortening of telomeres in T cell subsets from both diseases. Therefore, we measured the mean telomere length in the same cell

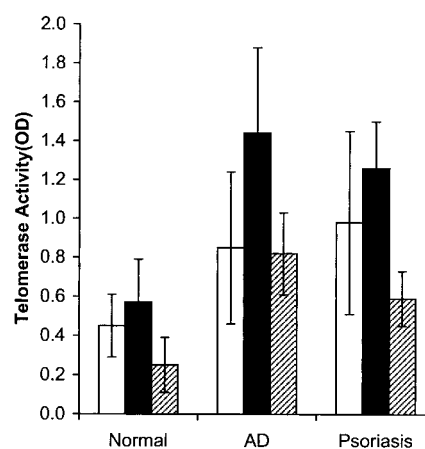


FIGURE 1. Increased telomerase activity was found in PBMC (□) and CD4⁺ (■) and CD8⁺ (▨) T cells from 10 healthy control persons, from 12 AD patients, and from 10 patients with psoriasis. Results are expressed as $\bar{x} \pm$ SD.

Table I. Mean TRF length (kilobases) determined in PBMC, CD4⁺, CD8⁺ T cells

Cell Source	Normal Control		Atopic Dermatitis		Psoriasis	
	TRF ^a	n ^b	TRF	n	TRF	n
PBMC	8.43 ± 0.82	26	6.95 ± 1.05 ^c	25	6.47 ± 0.78 ^c	15
CD4 ⁺	8.38 ± 0.52	10	7.05 ± 1.34 ^d	12	6.94 ± 0.73 ^c	10
CD8 ⁺	8.00 ± 0.60	10	7.06 ± 1.30 ^d	10	6.50 ± 1.10 ^d	6

^a Mean terminal restriction fragment length (mean ± SD).

^b Number of investigations.

^{c,d} Statistical significance for *t* test comparing the means of TRF among corresponding normal donors, atopic dermatitis patients, and psoriatic patients (^c *p* < 0.005, ^d *p* < 0.05). No significant difference was found between the atopic dermatitis and psoriasis groups.

populations in which we have measured telomerase activity. The results are presented in Table I. It is clearly shown that AD and psoriasis patients have significantly reduced telomeres of their lymphocytes in all subsets compared with normal persons (*p* < 0.05). We did not observe a difference of telomere length between CD4⁺ and CD8⁺ T cells.

To assess different subsets, we further measured the telomere length on CD4⁺ naive and memory T cells and on CD8⁺CD28⁺ and CD8⁺CD28⁻ T cell subsets in both diseases (Fig. 2 and 3). In addition to the fact that CD4⁺ memory T cells were found to have a significantly shorter telomere length than CD4⁺ naive T cells in AD (*p* < 0.01) and psoriasis (*p* < 0.001), we observed that CD4⁺ memory T cells and CD4⁺ naive T cells in both diseases have a significantly shorter telomere length than the same cell subsets from normal controls (*p* < 0.01). No significant difference was observed in telomere length between the CD8⁺CD28⁻ and CD8⁺CD28⁺ T cell subsets in both diseases. Interestingly, the telomere length of CD8⁺CD28⁺ T cells from AD and psoriasis was significantly shorter than that of the same cell population from normal donors (*p* < 0.01).

Telomerase activity and RNase inhibition

Some of the AD patients have eosinophilia, so we wished to determine whether this might influence our telomerase assay. It has recently been reported that eosinophils can secrete eosinophil-derived neurotoxin and eosinophil cationic protein, which are RNases that inhibit telomerase activity (26). We found two telomerase-negative AD patients with moderate eosinophilia in our data. When extracts from these two cases were mixed with extracts from telomerase-positive kidney cells (293 cells, 0.5 μg/assay) at room temperature before the telomerase PCR ELISA assay, the telomerase activity of the 293 cells was almost abolished. When we

added rRNasin (placental RNase inhibitor, 10 U/μl, Promega, Madison, WI) to the mixture of extracts described above, the telomerase activity of the 293 cells was rescued. We then prepared new extracts with the placental RNase inhibitor present in four telomerase-negative cases (two cases of AD with moderate eosinophilia and two cases of AD without eosinophilia as controls). The results showed that the two AD patients with moderate eosinophilia now expressed moderate telomerase activity, whereas the two AD patients without eosinophilia were negative for telomerase activity (Fig. 4).

Age effect on telomere length

It is well established that telomere length decreases with age. Thus, we performed a linear regression analysis, in which we looked for an age effect on telomere length of blood lymphocytes in both AD and psoriasis patients. The telomere length was 1.49 kb (95% CI = 1.06–1.91 kb) shorter among AD patients than among healthy controls. The telomere length was shortened 0.033 kb (95% CI = 0.015–0.050 kb) per year of age. The results showed that no interaction between age and AD was observed (*p* = 0.61). The telomere length was 1.52 kb (95% CI = 1.03–2.00 kb) shorter among psoriasis patients compared with that of healthy controls. The telomere length was shortened 0.030 kb (95% CI = 0.009–0.050 kb) per year of age. No interaction between age and psoriasis was observed (*p* = 0.51). The results confirmed that age is independent of the effects of the diseases. We also looked at eventual correlations between telomere length and total serum IgE level in AD patients and between telomere length and disease duration and intensity in psoriasis patients. However, we did not observe any significant correlations among these parameters (results not shown).

FIGURE 2. Comparison of mean telomere length on different T cell subsets from normal individuals (a) and from patients with psoriasis (b) and AD (c). In addition to the finding that CD4⁺ memory T cells have a significantly shorter telomere length than CD4⁺ naive T cells, we found that both of these cell subsets in diseases had a significantly shorter telomere than the same cell subsets from normal controls. The telomere length of CD8⁺CD28⁺ T cells from AD and psoriasis was significantly shorter than that of the same cell population from normal controls.

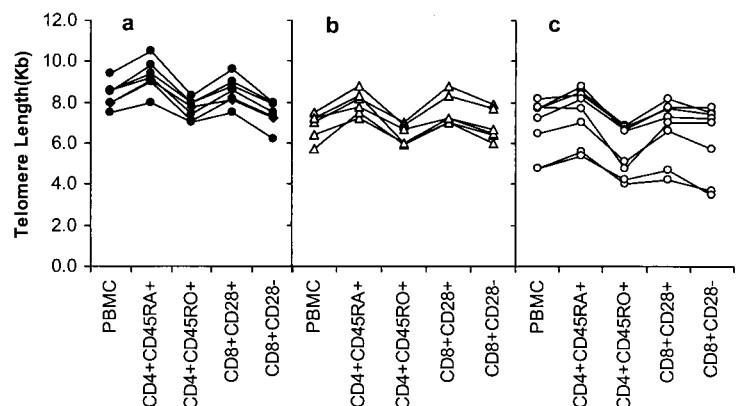
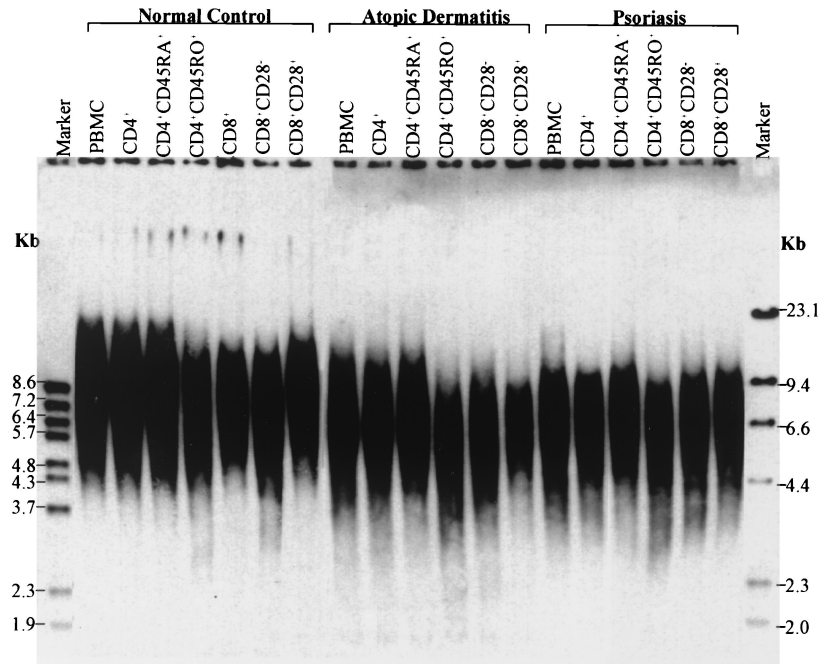


FIGURE 3. Southern blot analysis of mean telomere length in PBMC and CD4⁺, CD4⁺CD45RA⁺, CD4⁺CD45RO⁺, CD8⁺CD28⁻, and CD8⁺CD28⁺ T cells. Genomic DNA from normal controls and from patients with AD and psoriasis was digested with *RsaI* and *HinfI* and hybridized with biotinylated detection telomere probe; we then visualized the band on the film with chemiluminescence.



Discussion

AD and psoriasis are chronic inflammatory skin diseases, in which activated T cells are strongly implicated in the pathophysiological processes (27–29). This is indirectly supported by the fact that systemic use of cyclosporin, which specifically interrupts IL-2 production by activated T lymphocytes, will reduce or relieve both skin diseases (30, 31).

Previous studies have found that telomerase activity does not vary significantly as a function of the cell cycle of proliferating cells, but its activity is greatly reduced in quiescent cells (32). Freshly isolated lymphocytes show weak levels of telomerase activity as in our control persons, whereas ionophore or TCR stimulation leads to increased telomerase activity (17, 33). Therefore, it would be expected that PBMC of patients with AD or psoriasis show increased proliferation in vitro, but this is not the case (34). In this study we have demonstrated for the first time that both CD4⁺ and CD8⁺ T cells show increased telomerase activity together with a significant shortening of their telomeres.

We have performed a dilution test, in which we diluted the CD4⁺ T cells from four cases of AD patients with PBMC from normal controls in a range from 10% to 90% before performing the telomerase assay. Adding ~30% of AD CD4⁺ T cells to normal PBMC led to a significant increase in telomerase activity (data not shown). This could mean that a major portion of the cells had increased telomerase activity. We cannot exclude the possibility that it is a small subset of T cells that expresses a high telomerase activity, but if so, these cells must have a very high activity. It is hard to imagine that all “activated” T cells are driven by external Ag activation, because clonality has not been found in PBMC from patients with AD (35) or psoriasis (36). Thus, other factors are likely involved in the T lymphocyte “activation,” and our observations on reduced telomere length suggested that the T lymphocytes in both diseases are chronically stimulated. Although speculative, we have also considered whether there exist subpopulations of T cells at different developmental stages of T cell lineage than seen in normal persons (17, 37–39).

Increased telomerase activity should lead to preservation of the telomeres. Therefore, it was surprising to find that all subsets of T

cells in blood from both diseases had reduced telomere length, although CD4⁺CD45RA⁺ T cells had significantly longer telomeres than the CD4⁺CD45RO⁺ cells. Still, CD4⁺CD45RA⁺ cells from normal persons had significantly longer telomeres than the same cell subset from AD or psoriasis patients. Also, the CD8⁺CD28⁺ T cell population in AD and psoriasis patients had significantly reduced telomeres compared with control persons. This could mean an increased activity among the CD8⁺ cells, i.e., an increase in cytotoxic activity (40, 41). We could not show a

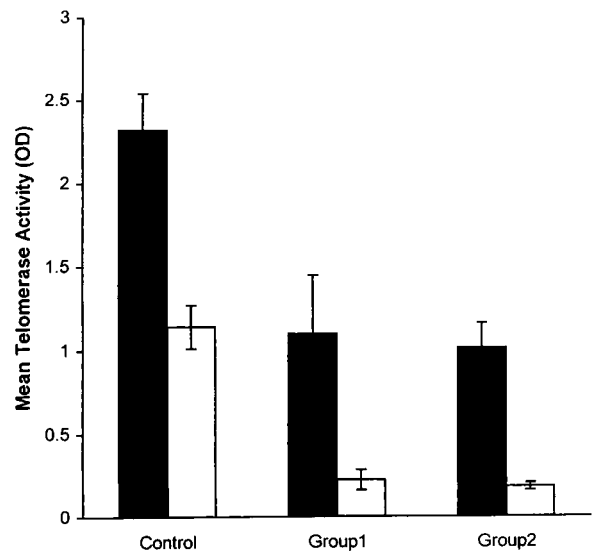


FIGURE 4. Influence of eosinophils on telomerase assay. Control: Telomerase-positive 293 cells at 4°C (■) or at room temperature (□) for 20 min before TRAP assay (0.5 μg/assay). Group 1: PBMC extracts from two cases with moderate eosinophilia were mixed with 293 cells at room temperature with (■) or without (□) placental RNase inhibitor (10 U/μl) before TRAP assay (0.5 μg/assay). Group 2: New extracts from these two cases were made at 4°C with (■) or without (□) placental RNase inhibitor before TRAP assay (1.0 μg/assay).

significantly shorter telomere length in CD8⁺CD28⁻ cells than in the CD8⁺CD28⁺ cell subset in diseased persons, as has been shown in HIV studies (42, 43), but we can observe a clear trend in this direction, and we speculated that perhaps the sample size has not reached statistical significance.

Our findings of the shortening of telomeres in T cells from AD and psoriasis patients suggest that a major subset of T cells is chronically stimulated in these patients and that these cells have likely gone through a larger number of cell divisions.

Recently, it has been shown that T cells from patients with HIV can have increased telomerase activity and reduced telomere length, although observations vary (44–46). It has also been found that telomeres shorten significantly during the first years of life (47). These as well as other studies have found that the telomeres shorten by 270 bp per year in children, whereas in adults it is ~50 bp per year.

We observed a difference between normal PBMC and AD PBMC of ~1400 bp and 1500 bp from psoriasis patients. The mean ages of control persons and of AD and psoriasis patients were 41, 37, and 47 years, respectively. Thus, these differences cannot be due to variation in age. If we calculate that 50 bp per year is the normal loss (47), it would mean that AD patients are, relatively, 28 years older, and psoriasis patients 30 years older, than healthy controls. Again, this very significant difference supports the view that T cells from these diseases are at a different developmental or biological stage than those from normal control persons.

It is noteworthy in this context that although both AD and psoriasis are quite different diseases from the clinical point of view, by and large they exhibit similar changes regarding their blood lymphocyte telomerase activity and telomere length. Our results also support the observation of Norrback et al. (26) that there is a strong correlation between the presence of eosinophilia and the absence of telomerase activity. The eosinophils are often increased in patients with AD and may affect the results. Therefore, it is advisable to add RNase inhibitors to the assay of the telomerase activity. We did not do so in every patient, but still we observed significantly increased telomerase activities.

In conclusion, our results demonstrate for the first time that T lymphocytes from blood of patients with AD and psoriasis have increased telomerase activity and reduced telomere length, indicating an *in vivo* activation of T lymphocytes. The shortening of telomeres is likely to reflect the increased cell proliferation, which would support the view that the affected T cells have gone through a large number of cell divisions because of chronic stimulation *in vivo*.

Acknowledgments

We thank Dr. Anne Braae Olesen (Department of Epidemiology, University of Aarhus) for performing a linear regression analysis.

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