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SENSITIVITY OF NATURAL KILLER CELLS TO ACTIVATION IN THE PROCESS OF AGEING IS RELATED TO THE OXIDATIVE AND INFLAMMATORY STATUS OF THE ELDERLY

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The role of the innate immunity during human ageing is not well understood. The aim of the study was to estimate reactivity of NK (natural killer) cells in the very old (mean age 91 years) and old subjects (mean age 78 years) compared to young individuals (mean age 26 years) in respect to the indices of the oxidative stress (telomere length of NK cells, serum content of -SH groups), serum total antioxidant status and serum concentrations of interleukin 6 and tumor necrosis factor- α (TNF- α). The activation state of NK cells, reflected by telomerase activity and intracellular interferon γ (IFN γ) content, was also measured. We found that length of telomeres in NK cells and serum concentration of -SH groups decreased both in the old and the oldest subjects as compared to young individuals. The oldest seniors, on the contrary to the old ones, revealed similar level of serum antioxidant status as the young subjects. The serum level of IL-6, not detectable in the young subjects, did not differ in the oldest and old seniors. TNF- α serum concentrations progressively increased with age. After stimulation, NK cells of both old groups showed higher intracellular levels of IFN γ than young subjects. IL-2-activated NK cells of the oldest seniors showed the highest increase of telomerase activity as compared to the other age groups. Serum level of IL-6 correlated positively with activation markers of NK cells. Moreover, in seniors but not in young subjects, the number of active, IFN γ -expressing NK cells, correlated positively with the serum content of the -SH groups. These findings indicate that sensitivity of NK cells to activation is maintained during ageing and this phenomenon may be related to the oxidative and inflammatory status of the elderly.

Key words: *ageing, inflammatory cytokines, natural killer cell activation, nonagenarians, oxidative stress, -SH groups, telomerase, telomeres*

INTRODUCTION

In contrast to other age-related phenomena, immunoageing is considered to represent rather a remodeling of the immune system than its progressive deterioration. The process of ageing mainly affects the adaptive part of the immune system as the innate compartment is relatively well preserved during ageing exemplified by an increase in the number of natural killer (NK) cells with normal cytotoxic activity (1). In general, immunosenescence is characterized by the involution of the thymus and the exhaustion of naive T cell population, decline of the absolute numbers of both T and B cells and deregulation of the Th1/Th2 system (2-4). Ageing is also accompanied by an imbalance between inflammatory and anti-inflammatory responses what results in the low grade chronic pro-inflammatory status called inflammageing (5, 6). This process is caused by a continuous, lifelong antigenic load and was proposed to be the most common and important cause of age-related pathologies (7).

Dysregulations of the immune functions normally are counterbalanced by continuous adaptation of the body to environmental challenges. Similarly, in healthy elderly the

elevated activity of NK cells may be regarded as a factor compensating for a decline of the number and function of T cells (3). NK cells are cytotoxic cells that play a critical role in innate immune response (8). Besides their cytotoxic function, NK cells secrete cytokines and chemokines that regulate the immunological reaction by promoting downstream adaptive, Th1 mediated response (9). Morphologically NK cells belong to a population of large granular lymphocytes defined by the CD3⁻, CD16⁺ and CD56⁺ surface phenotype. They constitute 10-20% of peripheral blood lymphocytes (10). It was found that NK activity well correlated with the health status of the elderly since healthy seniors revealed high cytotoxic NK activity until late senescence (11-13).

It has been established that important factors in cellular ageing such as shortening of telomeres, genomic instability, increased number of dysfunctional mitochondria and oxidative damage of cells result from the interactions of reactive oxygen species (ROS) with biological macromolecules. Dysfunction of mitochondria is in line with the free radical theory of ageing as the oxidative damage of mitochondria leads to the increased release of ROS. This oxidative damage could then be amplified and contribute to the age-related decline of mitochondrial

function and reduced energy production what finally affects cell functions (14, 15). Telomeres, complex DNA/protein structures at the ends of linear chromosomes, stabilize chromosomes by protecting DNA from degradation and recombination. However, during physiological ageing in various cell types telomeres become 'uncapped' what results in arrest of cell growth and initiates cell senescence (16). According to the telomere theory of ageing telomeres lose their protective function when they become too short (17). The telomere shortening is caused by the inability of DNA polymerases to completely copy the lagging strand (18). The oxidative stress and production of large amounts of ROS by damaged mitochondria are the main contributors to telomere shortening (19). Therefore, telomere length is considered to be a marker of both cellular ageing and oxidative stress (20, 21). However, age-related increase of oxidative stress may result also from a suppression of antioxidant defenses (15, 21) and telomere shortening rate was indeed shown to be also dependent on the antioxidant properties of cells (18, 19). Since chronic oxidative stress accompanies many inflammatory diseases, it was suggested that telomere length may be regarded as an indirect marker of inflammatory process (4, 20).

Studies on human longevity performed by Olshansky *et al.* revealed that in humans the age of 85 years may be regarded as the average biological life limit (22). Nonagenarians and centenarians represent a unique population of seniors since they often present successful ageing *i.e.* a good psychophysical status (7). Although many apparently healthy centenarians reveal high serum concentrations of pro-inflammatory cytokines (23), they also present unchanged number of T lymphocytes, increased production of immunoglobulins, lack of organ-specific autoantibodies, well preserved NK activity and normal proliferative potential of T lymphocytes (24).

It has been suggested that oxidative stress may also contribute to the age-related changes of the immune system (25), however, this subject has yet not been investigated in humans. Therefore, we decided to define relationships between serum markers of oxidative and inflammatory status as well as the activation state of the NK cells in a well characterized group of young and two groups of old subjects: nonagenarians who represented a population of selected, very long-lived seniors and the elderly below the critical age of 85. We also analyzed the correlations between both oxidative and inflammatory status and biomarkers of the NK cell activation (26).

MATERIAL AND METHODS

Study population

Peripheral blood mononuclear cells (PBMC) were obtained from 32 young subjects referred to as 'young' (mean age 26±5 years, S.D.; 16 women and 16 men) and 32 elderly: 23 seniors at the age below 85 referred to as 'old' (mean age 78±5 years; 19 women and 4 men) and 9 seniors at the age over 85 referred to as 'the oldest' (mean age 91±4 years; all women). All subjects lived in urban area. The volunteers did not suffer from infections, inflammatory or autoimmune diseases for six months before and during the study. However, some seniors were afflicted with degenerative joint and spine disease (14 subjects), cataract (3 subjects), depression (6 subjects), circulatory insufficiency (4 subjects), ischemic heart disease (10 subjects), ischemic brain disease (1 subject), diabetes mellitus (3 subjects), osteoporosis (3 subjects). Seniors suffering from degenerative joint and spine disease, cataract and osteoporosis did not take any medications. Seniors suffering from depression took antidepressants. Seniors suffering from circulatory insufficiency took vasodilators and digitaloids. Seniors suffering from

ischemic heart disease took vasodilators, antithrombotic and diuretic drugs. Seniors suffering from diabetes mellitus took antidiabetic drugs. The senior suffering from ischemic brain disease took a cerebral vasodilator.

Informed consent from volunteers and approval from Ethical Committee of Medical University of Gdansk were obtained.

Peripheral blood mononuclear cell separation

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by conventional ficoll-uropline density gradient centrifugation. PBMC were collected at the interphase, washed and resuspended in PBS-1% FBS solution.

Natural killer cell separation

Natural killer (NK) cells (CD3⁺CD16⁺CD56⁺) were isolated from PBMC by negative selection with the use of NK isolation kit and MACS magnetic separator (Miltenyi Biotec, USA). PBMC were incubated with biotin-antibody cocktail (suspension of biotin-conjugated monoclonal antibodies against CD3, CD4, CD14, CD15, CD19, CD36, CD123 and glycophorin A) for 10 min, then washed with washing buffer and incubated with anti-biotin microbeads (microbeads conjugated to monoclonal anti-biotin antibodies) for subsequent 15 min. Then cells were resuspended in 500 µl of washing buffer and applied onto the column. Cells eluted from the column in magnetic field represented the enriched NK cell fraction of about 90% purity. The purity of the enriched NK cells was evaluated by flow cytometry. Aliquots of the cell fractions were stained with anti-CD56 PE-conjugated (BD, PharMingen, USA) and anti-CD3 FITC-conjugated (BD PharMingen, USA) monoclonal antibodies (20 µg/test).

Telomere length determination in natural killer cells by flow-FISH method

Telomeres were detected with the use of telomere PNA Kit/FITC for flow cytometry (DAKO, Denmark). Briefly, purified NK cells (1x10⁶ cells) were suspended in 300 µl of hybridization mixture containing 70% formamide with (test samples) or without (control samples) FITC-conjugated telomere PNA (peptide nucleic acid) probe (CCCTAA)₃. Samples were heat-denatured at 82°C for 10 min and hybridised at room temperature in the dark overnight. Then cells were washed twice with Wash Solution and stained with propidium iodide solution (10 µg/ml propidium iodide and 100 µg/ml RN-ase in PBS) for 2 hours. Flow cytometric analysis was performed on Coulter Epics XL cytometer equipped with 488 nm argon laser. The obtained data were used for the determination of a relative telomere length (RTL), *i.e.* telomere length of sample cells compared to telomere length of control cells (1301 cell line characterized by very long telomeres) (27).

Estimation of telomerase activity in natural killer cells

Telomerase activity was measured in NK cell extracts with the use of TRAP_{EZE}[®] ELISA Telomerase Detection Kit (Chemicon International, USA) following manufacturer's instructions. NK cells freshly isolated or activated with IL-2 (500 U/ml, 96 h) were suspended in lysis buffer containing CHAPS on ice for 30 min. After centrifugation supernatants were transferred into fresh tubes, quick-frozen in liquid nitrogen and stored in -70°C until use. TRAP (Telomeric Repeat Amplification Protocol) reaction consisted of 2 steps: in the first one telomerase present in extracts added a number of telomeric repeats (GGTTAG) to the 3' end of biotinylated telomerase

substrate oligonucleotide (b-TS) and in the second step the extended products were amplified by PCR. As the TRAP extension/amplification reaction was performed with biotinylated primers and DNP (dinitrophenyl)-labeled dCTP, the TRAP products were tagged with biotin and DNP residues. The labeled products were immobilized to streptavidin-coated microtiter plates *via* biotin-streptavidin interactions and then detected by anti-DNP antibodies conjugated to horseradish peroxidase (HRP). The amount of TRAP products was determined by HRP activity with the use of TMB substrate (3,3',5,5' tetramethylbenzidine) and subsequent color development. Absorbance of the samples was measured at 450 nm (with a reference wavelength of 690 nm) using Bio-Rad microplate reader.

Determination of the number of interferon γ -expressing natural killer cells

PBMC (1×10^6 cells/ml RPMI1640 containing 5% FBS) were activated 5 hours with PMA (phorbol 12-myristate 13-acetate) and ionomycin both supplied by Sigma, USA and used at final concentration of 50 ng/ml. Simultaneously 2 μ l/well of Golgi-Stop reagent (BD PharMingen, USA) was added to PBMC cultures to stop extracellular export of interferon γ (IFN γ). Additionally, non-stimulated cultures were performed for the measurement of spontaneous release of intracellular IFN γ . After 5 hours PBMC were collected, washed with PBS (Ca²⁺ and Mg²⁺ free) containing 1% FBS and 0.09% sodium azide and stained for subsequent 30 min with anti-CD3 ECD-labeled (Immunotech Beckman Coulter, France) and anti-CD56 PE-labeled monoclonal antibodies (BD PharMingen, USA). Then, cells were washed and fixed in 4% paraformaldehyde (Sigma, USA) in PBS (Ca²⁺ and Mg²⁺ free), washed and permeabilised for 20 min using 0.1% saponin in PBS (Ca²⁺ and Mg²⁺ free) containing 1% FBS and 0.09% sodium azide. After permeabilisation cells were washed, stained with anti-IFN γ FITC-labeled monoclonal antibodies (1 μ g/test) (BD PharMingen, USA) and incubated for 30 min. Appropriate isotype controls were also prepared. Then samples were washed, fixed in 2% paraformaldehyde and analyzed by flow cytometry with the use of Coulter Epics XL cytometer equipped with 488 nm argon laser.

Determination of the total -SH group content in serum

The total -SH group content in serum was measured with DTNB (2,2-dithio-bis-nitrobenzoic acid) assay. 460 μ l of sodium phosphate buffer (10 mM) pH 8.0 and 290 μ l of water was added to each tube. After addition of 100 μ l of sodium dodecyl sulphate (10%) to expose hidden sulphhydryl groups of proteins, 50 μ l of serum was added and vigorously mixed. Subsequently, 100 μ l of DTNB (1 mM) was added into the experimental sample (prepared in duplicates) or 100 μ l of sodium phosphate buffer into the reference sample. The samples were then incubated at 37°C for 1 hour and absorbance was measured at 412 nm with Bio-Rad plate reader. A standard curve reflecting absorbance of the increasing concentrations of a reduced form of glutathione (GSH, Sigma) was prepared and used to measure the content of sulphhydryl groups in the experimental samples. Data are presented as nmoles of -SH groups/mg of serum protein in a 50 μ l sample. Serum protein concentration was measured according to Bradford.

Determination of total antioxidant status in serum

Total antioxidant status (TAS) was measured with the use of Cayman antioxidant assay (Cayman Chemical Company, USA)

following manufacturer's instructions. The assay is based on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate) to ABTS⁺ by metmyoglobin. The amount of ABTS⁺ is then measured by reading the absorbance at 405 nm. The suppression of the absorbance is proportional to the concentration of antioxidants in the sample. The capacity of serum antioxidants is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as millimolar Trolox equivalents. Briefly, 150 μ l of chromogen, 10 μ l of metmyoglobin and 10 μ l of serum or Trolox standard, respectively, were placed in the designated wells on a microtiter plate. To initiate the reaction, 40 μ l of hydrogen peroxide working solution was added to all plate's wells. The plate was incubated on a shaker for five min at room temperature and then the absorbance was read at 405 nm using Bio-Rad plate reader. Total antioxidant status of the samples was determined with the use of a standard curve prepared for Trolox and presented as molar concentration of antioxidants in serum (mmol/l).

Determination of cytokine concentrations in serum

The human IL-6 immunoenzymatic assay (ELISA) was carried out according to the protocol delivered by the producer (R&D Systems, Europe, United Kingdom). 100 μ l of assay diluent was pipetted into 96 wells of the microtiter plate coated with a mouse monoclonal antibody against IL-6. Then 100 μ l of standard, control or serum was added to each well. After 2 hours of incubation at room temperature culture medium was aspirated and the plate was washed four times. Next, 200 μ l of IL-6 conjugate was added to each well and after two hours medium was aspirated and the plate was washed four times. Then 200 μ l of substrate solution was added to each well. After 20 min of incubation at room temperature in the dark the reaction was stopped by pipetting to each well 50 μ l of stop solution. The absorption was read at 450 nm with plate reader (Bio-Rad).

The human TNF- α immunoenzymatic assay (ELISA) was carried out similarly to that of IL-6 described above according to the protocol delivered by the producer (R&D Systems Europe, United Kingdom).

Statistics

Statistical analysis was performed with the use of STATISTICA 8.2 program. Parametric and non-parametric distributions were calculated by W Shapiro-Wilk test. The analyzed data were then analyzed by non-parametric Kruskal-Wallis test to compare three age groups or U-Mann-Whitney test to compare two age groups. Wilcoxon test was used for comparisons of differences between two related samples. Differences with $p < 0.05$ were considered as statistically significant. The experimental data are presented in the study as median and percentiles (25%/75%).

RESULTS

Analysis of selected parameters of oxidative stress, total antioxidative capacity and inflammatory status in sera of young, old and the oldest subjects

The serum content of protein sulphhydryl groups was higher in the group of young subjects as compared to both senior populations which presented similar -SH group levels (Fig. 1A).

The young population had also higher serum total antioxidant capacity (TAS) than the group of old subjects (Fig.

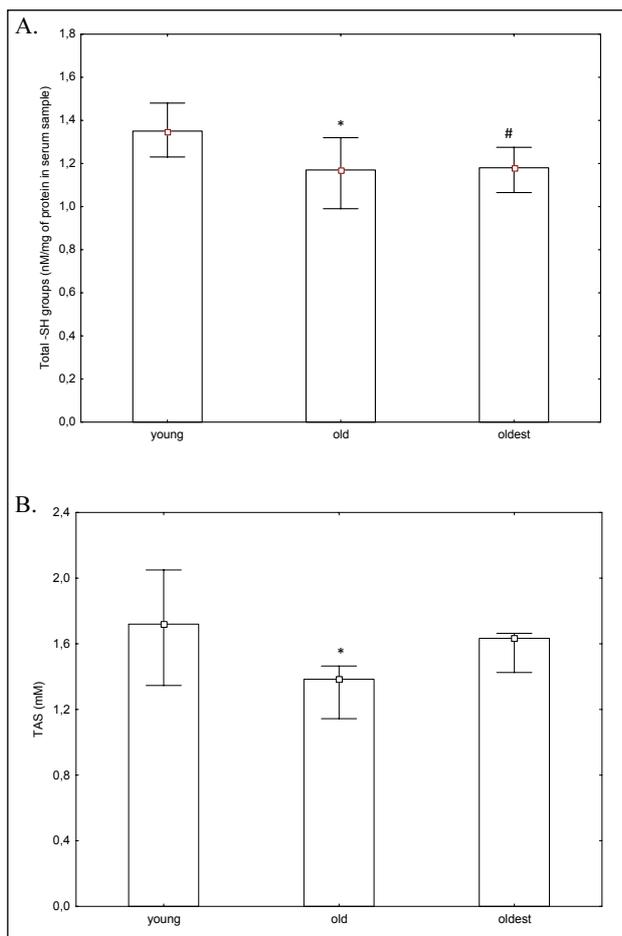


Fig. 1. Parameters of oxidative/reductive status in sera of young, old and the oldest subjects. *A.* Serum content of sulphydryl (-SH) groups presented in nmoles/mg of protein in serum sample. *B.* Total antioxidant capacity (TAS) of serum presented as molar concentration of antioxidants in serum (mM). Data are shown as median values and 25th/75th percentiles. * young vs. old $p < 0.05$; # young vs. the oldest $p < 0.05$.

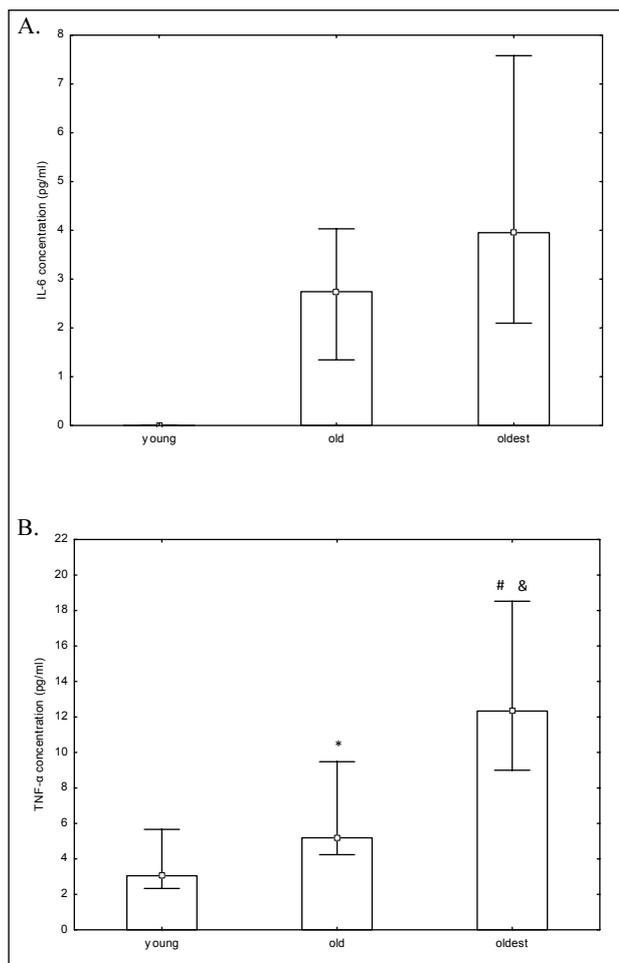


Fig. 2. Concentrations of inflammatory cytokines in serum. *A.* Interleukin 6 levels. In the young group IL-6 was not detected. The difference between two old groups was not statistically significant. *B.* TNF- α levels. Data are shown as median and 25th/75th percentiles. * young vs. old $p < 0.05$; # young vs. the oldest $p < 0.001$; & old vs. the oldest $p < 0.01$.

IB). However, serum TAS of the oldest seniors did not differ significantly from that of young subjects.

IL-6 levels were detected only in the sera of the old and the oldest subjects. Both groups of seniors had similar concentrations of IL-6 (*Fig. 2A*). The oldest seniors presented significantly higher serum TNF- α concentration than the old and young subjects. Serum TNF- α level was also significantly higher in the old as compared to the young population (*Fig. 2B*).

Telomere length of natural killer cells and its relation to the indices of oxidative stress and inflammatory status

Telomeres of the NK cells of the elderly were significantly shorter than telomeres of the young persons. However, telomere length was similar in the NK cells of the old and the oldest subjects (*Fig. 3*).

We did not find any changes in the examined parameters between women and men and between seniors afflicted with some diseases and seniors who did not suffer from these diseases (data not shown).

When the levels of total serum -SH groups of all investigated individuals were plotted against the telomere length of NK cells,

we found a highly significant positive correlation (*Fig. 4A*). We observed also a negative correlation between telomere length of NK cells and serum TNF- α concentration of all investigated subjects (*Fig. 4B*). However, there was no correlation between telomere length and serum levels of IL-6 (data not shown).

Sensitivity of natural killer cells to activation

When PBMC isolated from all age groups were stimulated for 5 hours with PMA and ionomycin to estimate their ability to produce intracellular IFN γ , the NK cells of both the old and the oldest seniors showed significantly higher increase in the intracellular IFN γ levels than young subjects. The difference between the old and the oldest subjects was statistically not significant. The control, unstimulated PBMCs of all age groups did not show detectable levels of IFN γ (*Table 1*).

All three age groups showed a significant increase in the telomerase activity after stimulation of the NK cells with IL-2. Although the differences between the age groups were statistically not significant, the oldest seniors showed tendency to present the highest increase of the telomerase activity in relation to control, unstimulated cells (*Table 1*).

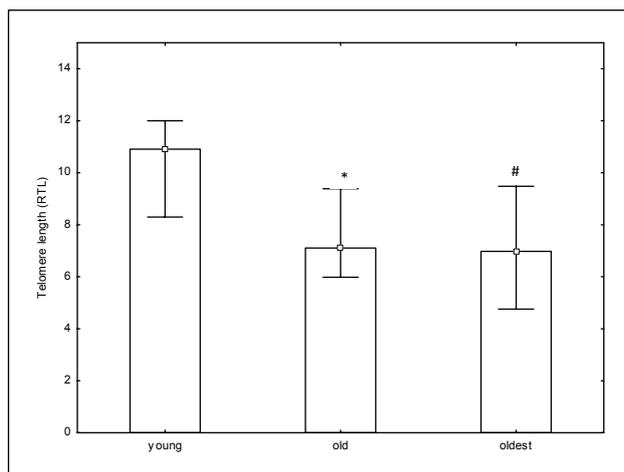


Fig. 3. Telomere length of the NK cells. Telomere length is expressed in relative telomere length units (RTL). Data are shown as median and 25th/75th percentiles. * young vs. old $p < 0.001$; # young vs. the oldest $p < 0.01$.

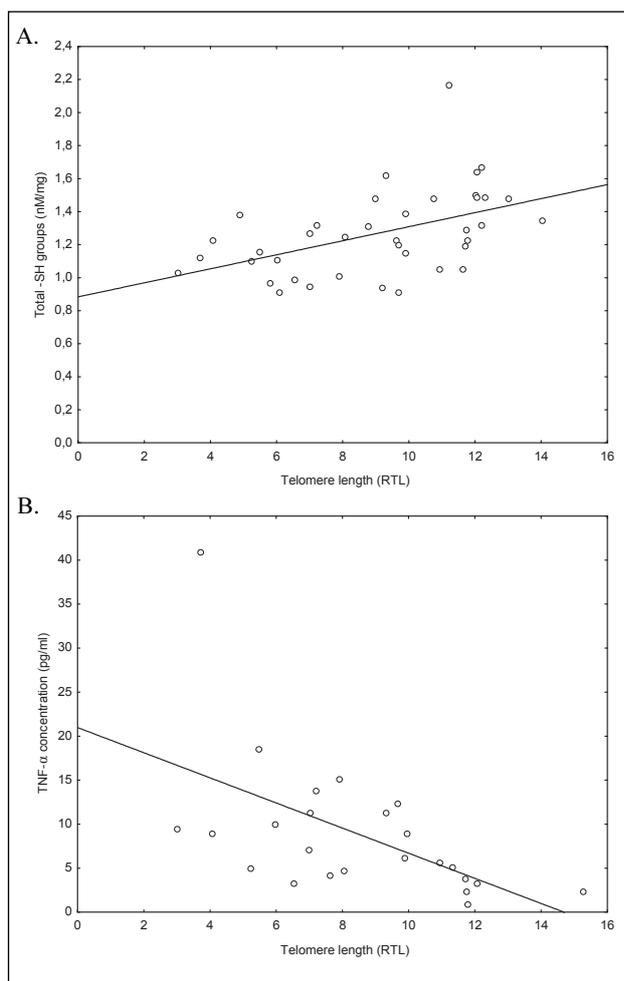


Fig. 4. Correlations between telomere length and parameters of oxidative stress and inflammatory status ("n" corresponds to a number of valid cases). A. A positive correlation between telomere length (RTL) and content of sulphhydryl groups (Total-SH groups) in sera of all subjects ($r=0.480$; $p=0.002$; $n=39$). B. A negative correlation between telomere length (RTL) and concentration of TNF- α in sera of all subjects ($r=-0.541$; $p=0.006$; $n=24$).

Table 1. Parameters of the NK cell activity in the young, old and the oldest subjects presented as the expression of intracellular IFN γ and telomerase activity. Expression of intracellular IFN γ is shown as the percentage of CD3-CD56+IFN γ + cells after stimulation of NK cells for 5 hours with PMA and ionomycin (50 ng/ml). Control, unstimulated NK cells did not express IFN γ . Telomerase activity (in arbitrary units) was measured after incubation of NK cells for 96 hours without (control) and with IL-2 (500 U/ml). The results are shown as median and percentile (25%/75%) values. The differences between age groups were estimated by U-Mann-Whitney test. *,# $p < 0.05$ vs. young group. Differences between levels of telomerase activity in control and IL-2-stimulated NK cells in the same age group were assessed by Wilcoxon test. $\blacktriangle p < 0.001$ and $\& p < 0.05$.

Parameter	Age group		
	young	old (seniors < 85)	oldest (seniors > 85)
Percentage of NK cells with expression of intracellular IFN γ	1.259 0.656/2.736	2.930* 1.176/10.187	3.459# 2.315/6.083
Telomerase activity in non-stimulated NK cells	0.023 0.018/0.049	0.028 0.017/0.123	0.016 0.013/0.072
Telomerase activity in IL-2-stimulated NK cells	0.081 \blacktriangle 0.047/0.702	0.135 \blacktriangle 0.045/0.905	0.364 $\&$ 0.354/0.482

Correlations between natural killer cell activation and oxidative and inflammatory status

We found a strong positive correlation between the percentage of IFN γ -expressing NK cells and content of sulphhydryl groups in sera of both groups of seniors (Fig. 5A). However, no correlation was observed in the group of young subjects (data not shown).

A positive correlation was found between telomerase activity of IL-2-activated NK cells and serum IL-6 concentration in all age groups (Fig. 5B). There was also a positive correlation between the percentage of IFN γ -expressing NK cells and serum IL-6 concentration in all investigated individuals (Fig. 5C). Serum concentrations of TNF- α did not correlate with indices of the activation of NK cells in none of the age groups (data not shown).

DISCUSSION

Ageing is accompanied by the alterations of both adaptive and innate immunity. The increase of serum levels of IL-6, TNF- α and other inflammatory mediators results in a state of a chronic low-grade inflammation. This process, called inflammageing (6, 24) is probably caused by increased antigenic load throughout life and results in the progressive activation of leukocytes including macrophages and NK cells. Although it was suggested that oxidative stress may also contribute to the age-related changes of the immune system (25), the lack of human studies prompted us to define relationships between serum markers of oxidative and inflammatory status as well as the activation status of the NK cells human ageing.

Harman first proposed that free radicals produced during aerobic metabolism are likely key factor involved in the ageing process since they cause cumulative oxidative damage of macromolecules and cellular structures (28). During the last 15 years the free-radical theory of ageing had been widely tested and gained substantial support from molecular and cellular biological studies of ageing (14, 29). Although pro-oxidants may come from many sources in the mammalian cell, mitochondria have been established as the major generator and direct target of ROS

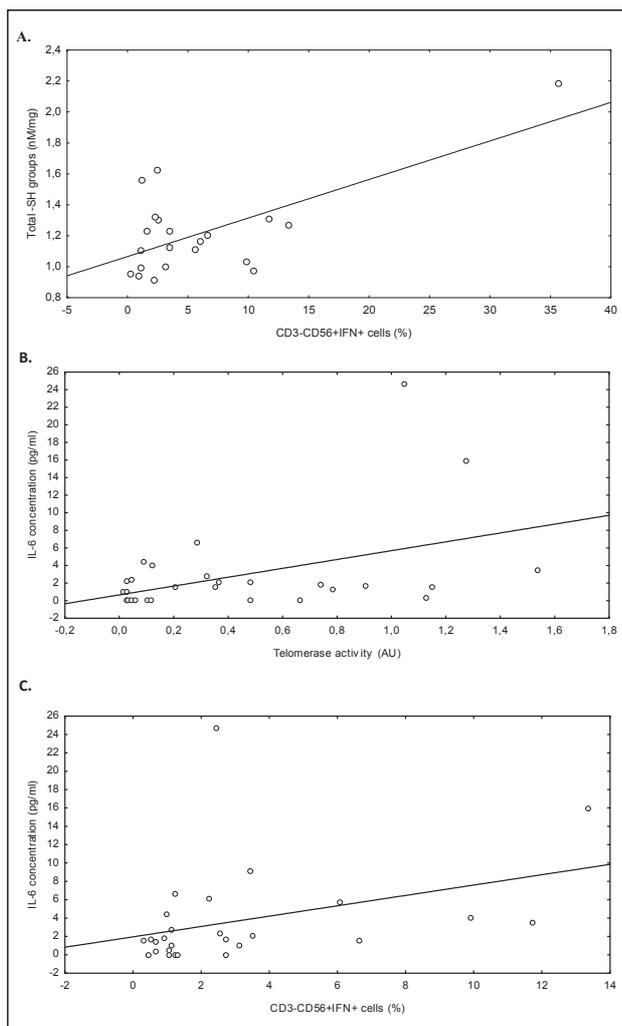


Fig. 5. Correlations between parameters of the NK activation and indices of oxidative stress and inflammation (“n” corresponds to a number of valid cases). *A.* A positive correlation between the percentage of intracellular IFN γ - expressing NK cells (CD3-CD56+IFN γ + cells) and content of -SH groups in sera of all seniors ($r=0.666$; $p=0.001$; $n=21$). *B.* A positive correlation between telomerase activity level in activated NK cells and concentration of IL-6 in sera of all subjects ($r=0.441$; $p=0.015$; $n=30$). *C.* A positive correlation between the percentage of intracellular IFN γ expressing NK cells (CD3-CD56+IFN γ + cells) and IL-6 concentration in sera of all subjects ($r=0.610$; $p=0.001$; $n=26$).

oxidative damage (14, 15). Decline in the function of electron transport chain in mitochondria results in reduced capacity to produce ATP in the tissues of old mammals (30). Moreover, decreased mitochondrial biogenesis in ageing also contributes to cellular energy deficits (31). Lower capacity to generate cellular energy may lead to diminished resistance of older individuals to oxidative stress as compared to younger subjects (32).

However, the age-related increase in oxidative stress may also result from a decline of antioxidant defense systems which operate in a balanced and coordinated way, and each component definitively relies on the action of the others (14, 15). The antioxidant enzymes, superoxide dismutases (CuZnSOD and MnSOD), catalase, and glutathione peroxidase, belong to the key components of the antioxidative barrier (33). It has been proven that in the process of ageing the efficacy of anti-oxidative

enzymes decreases (15). Additional role is played by the non-enzymatic antioxidative components such as macromolecules (albumins, ceruloplasmin, ferritin) and many small molecules (ascorbic acid, α -tocopherol, β -carotene, reduced glutathione, uric acid, bilirubin) (33). The complexity of the antioxidant defenses is highly increased by the intracellular or extracellular location of its components, as well as tissue, gender and species specificity (15, 34, 35). Moreover, similarly to some other important cellular processes, there is a clear compartmentalization of antioxidant enzymes and non-enzymatic antioxidants (15, 33). *E.g.* mitochondria have their own GSH (reduced glutathione) pool independently of the cytosol (36), and the cell nucleus has its own set of antioxidative enzymes and compounds (37). During ageing a pro-oxidant shift in intracellular glutathione redox state occurs (38), *e.g.* in mouse an increase in oxidized glutathione (GSSG) and a decrease in reduced glutathione (GSH) levels in all tissues was observed (39).

The mitochondrial production of superoxide anions is the major source of ROS and effective antioxidant mechanisms evolved to neutralize their harmful effects. Superoxides in the matrix of mitochondria are dismutated by matrix MnSOD, anions released to the intramembrane space are partly dismutated by space CuZn-SOD, and superoxides which diffuse into the cytosol are converted by cytosolic CuZnSOD (15). If any mitochondrial reactive oxygen species reach the extracellular space, they are detoxified by extracellular (plasma) CuZnSOD (33). The studies of the activities of SOD isoenzymes in ageing provided inconsistent results depending on the species, tissue and experimental methods (15, 39). Moreover, the antioxidative activities of human catalase and glutathione transferase, two other basic components of antioxidative defense, decrease with age (40). Investigations of the non-enzymatic antioxidative systems provided inconclusive data. *E.g.* the levels of glutathione, ascorbic acid, albumin and selenium decrease and the level of ceruloplasmin and uric acid increase during ageing (35, 39). However, the level of antioxidants may also depend on their dietary supply which was shown to vary significantly between human subjects during ageing (41). Generally, the available data suggest that during ageing the balance of oxidant and antioxidant systems both inside and outside the cells shifts in favor of accelerated oxidation.

The previous paragraphs document not only the complexity of the oxidative/antioxidant mechanisms in ageing but also inevitable problems encountered by investigators of human ageing. For ethical reasons only a few human tissues and cells like erythrocytes (42, 43), plasma or serum (44, 45), and in rare cases muscle biopsies (46, 47) may be used for the investigations of the oxidative status of the elderly. Since oxidative stress decreases serum concentration of thiol groups and shifts the thiol/disulfide redox balance to the more oxidative state (48), serum content of sulphhydryl groups (-SH groups) is considered to reflect the level of oxidative stress (49). Similarly to other authors (44, 45, 50, 51) we also found decreased serum content of the -SH groups in both groups of the elderly. Although there are many potential causes for the age-related increase in the oxidative stress, one important factor, albeit often overlooked, level of may be represented by decreased antioxidative capacity (52). The sum of the activities of both enzymatic and non-enzymatic antioxidative systems represents the total antioxidant activity (49). Usually, plasma or serum antioxidants are not measured individually because of various sensitivity of the detection techniques and multiple interactions between different components of the antioxidant system. Therefore, measurements of the total antioxidant status (TAS) of plasma/serum better characterize body's oxidative defenses than the analysis of one or few antioxidative factors (49, 51, 52). Such approach enabled us to show that in respect to the oxidative status the elderly did

not represent a homogenous group since the oldest, but not the old individuals, revealed level of the antioxidant capacity similar to the young subjects. Environmental factors could affect findings of other authors who showed that in Mexico's rural areas TAS was comparable between adults (mean age 33±6.4 years) and seniors (mean age 70±8.3 years), however, in urban areas the elderly (mean age 68±7 years) presented lower serum antioxidant capacity than the adults (53). Our study is the first one in which effect of ageing on the level of oxidative stress was assessed in two groups of the elderly who were on average by 15 and 30 years older than the sexagenarians or septuagenarians investigated by other authors (52-55). Results of this study do not support the notion that physiological ageing is accompanied by a steady progressive decline of the antioxidant capacity (55) since in our study the oldest elderly had, in contrast to septuagenarians, similar TAS as the young subjects. Our results suggest that active antioxidant mechanisms may constitute an important marker of successful ageing.

Immunageing is characterized also by a complex remodeling of cytokine production expressed by increased plasma levels of pro-inflammatory mediators such as IL-1, IL-6 and TNF- α (7, 23, 24). However, the ageing process in a different way affects production of various types of proinflammatory cytokines since in our study TNF- α serum concentration was significantly higher in nonagenarians than in septuagenarians, whereas IL-6 serum level was similar in both groups of the elderly. Although the measurements of cytokine serum levels do not allow to define their cellular origin, our results suggest that in the very old subjects TNF- α concentration may better than the IL-6 level reflect the state of low grade chronic pro-inflammatory status defined as inflammageing (6).

Shortening of telomeres is regarded as the universal feature of the ageing process. Similarly to other authors (56) we also found significantly shorter telomeres of the NK cells in the elderly. Since oxidative stress is regarded as an important factor in the development of inflammatory reaction and a main contributor to the process of telomere shortening, telomere length may be considered to be a marker of both the oxidative stress and inflammatory state (16-20). Our findings confirm this suggestion since we found a positive correlation between telomere length of NK cells and serum content of sulphhydryl groups, which both decreased with ageing. The results of our study also revealed the negative relationship between telomere length and serum concentration of TNF- α , a marker of the inflammageing process.

Telomerase activity is substantial for the maintenance of the proliferative capacity and clonal expansion of activated lymphoid cells (57-59). Since IL-2 is a very potent immunomodulator acting on various lymphocyte populations including NK cells (60, 61), we compared telomerase activity in IL-2 activated NK cells isolated from the analyzed populations. Our studies showed that after stimulation of NK cells with IL-2, all three age groups revealed increased telomerase activity in stimulated calls. No significant differences of the telomerase activity of activated NK cells were observed between the age groups, although the highest increase of the enzyme activity was observed in the NK cells of the oldest seniors. Thus, the previously observed by us (26) and others (56) decline of the telomerase activity in resting NK cells of the elderly, could be completely reversed by the activation of the cells with IL-2, their natural ligand.

In recent years it became clear that the NK cells besides functioning in the destruction of virus-infected or transformed cells may also promote recruitment and response of the other cells of the immune system by the release of cytokines and chemokines (62). IFN γ is an important cytokine secreted by various cell types at the initial phase of immune response involved in the activation and differentiation of T cells, B cells,

NK cells and macrophages. As NK cells secrete IFN γ only after activation it is regarded to be a marker of activated NK cells (63). IFN γ enhances the differentiation of Th1 cells and maintains the cytotoxic function of NK cells and T cells (6). Thus, by secreting IFN γ NK cells may play an important role in linking innate and adaptive immunity (62, 65). Our results suggest that ageing does not suppress the ability of IL-2 activated NK cells to synthesize IFN γ since the percentage of IFN γ -expressing NK cells was significantly higher in the elderly as compared to the young subjects. Miyaji *et al.* also observed the prominent increase of IFN γ production by activated NK cells of centenarians compared with the middle-aged (42-64 years) subjects (64). The increased number (2) and functional capacity of the NK cells of the elderly humans may compensate for their lower proliferation and production rate in healthy old subjects (66).

We were able to demonstrate novel observations on the relationships between oxidative stress, inflammation and activity of NK cells in the context of the ageing process. The strong positive correlation between serum levels of sulphhydryl groups and levels of intracellular interferon γ in NK cells suggests that seniors with lower level of oxidative stress (reflected by higher content of -SH groups in serum) had NK cells more sensitive to the activation. Conversely, these data suggest that increased oxidative stress may compromise functional capacity of the NK cells not only during ageing but probably also during states associated with the disturbances of the redox homeostasis.

The positive correlations between serum level of IL-6 and both telomerase activity and expression of intracellular IFN γ in activated NK cells of young and old subjects suggest that activation of the NK cells is accompanied by a low-grade chronic inflammatory state characteristic for the ageing process. The relatively small in absolute values, albeit highly significant, increased serum concentration of inflammatory cytokines present in the elderly may reflect an adaptation of the innate immunity to the higher level of an "immunological stand-by" that enables effective responses to the antigenic stimulation. Any disturbances of this inflammatory/anti-inflammatory balance that may result from changes in oxidant/antioxidant status may also increase the susceptibility to age-related diseases or disabilities. Thus, successful ageing seems to be based on both the balance between pro- and anti-inflammatory processes and efficient antioxidative defenses.

Results of our investigations provide further evidence that centenarians and nonagenarians belong to the special group of the elderly who reach the extreme limit of human life in good clinical conditions despite being subjected to the process of immunosenescence.

In conclusion, we have demonstrated that healthy ageing of nonagenarians was associated with high activity of the NK cells that was related to the oxidative-inflammatory status. Measurements of the serum indices of the oxidative state, levels of proinflammatory cytokines and NK cell activity may prove useful data to assess successful ageing.

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