

Psychoneuroendocrine correlates of lymphocyte subsets during healthy ageing

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Abstract

Ageing has been associated with increased cortisol levels and absolute counts of T lymphocytes with memory phenotype. Although the mechanisms underlying these changes are still unknown, it has been speculated that this could be related to a dysfunction in FAS/CD95 expression in naive or memory cells. In this study, we investigated the role of psychoneuroendocrine variables in regulating CD95 expression on lymphocyte subsets. Forty-six elderly subjects (65–91 years) and 33 young adults (20–40 years) were recruited according to the SENIEUR protocol. The psychological status was measured by structured clinical interviews, salivary cortisol was assessed along the day (9, 12 and 22 h) and peripheral blood lymphocytes were immunophenotyped. The elderly were more stressed, depressed and anxious than the young subjects. Cortisol levels were increased in the elderly, indicating an activation of the hypothalamic–pituitary–adrenal (HPA) axis. We observed reduced counts of CD45RA+CD95+ cells in the elderly compared to young adults. The elderly subjects also showed a reduced expression of CD3 and CD62L in contrast to increased CD95 expression in CD45RA+ cells. The emotional state was positively correlated with the lymphocyte markers. Our data suggest the healthy ageing is associated with psychoneuroendocrine alterations that may be implicated in the regulation of CD95 expression on peripheral T cells.

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1. Introduction

Ageing has been associated with several dynamic immunological alterations which are collectively called immunosenescence. These changes include thymic involution, impaired humoral responses, intact innate immunity and reduced cell-mediated immunity (Pawelec et al., 2002). The latter can be demonstrated by a blunted T-cell proliferation, altered cytokine production and changes in lymphocyte distribution. It has been demonstrated that ageing is associated with significant changes in peripheral T-cell subsets (Pawelec et al., 2002). In particular, it was observed a relative decrease in peripheral CD4+ cells compared to

CD8+ cells in old rats (Miller et al., 1997). In addition, several studies observed a decrease in naive (CD45RA+) with respective increase in memory T cells (CD45RO+) (Hannet et al., 1992; Gabriel et al., 1993). However, the physiological mechanisms that regulate the proportion of peripheral naive/memory cells are largely unknown. It was recently proposed that the accumulation of memory T cells during ageing could be due to increased expression of CD95 (APO1/Fas) in naive cells, augmenting its sensitivity to apoptosis (Potestio et al., 1999). CD95 is a member of tumor necrosis factor (TNF) family and its ligand (CD95L) is found on activated T cells (Nagata and Golstein, 1995). The CD95–CD95L binding seems to play an important role in maintaining the cellular homeostasis of the immune system and may contribute to stress-related immunological changes (Yin et al., 2000). However, the role of the psychoneuroendocrine factors in regulating Fas

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expression on lymphocytes during ageing remains to be determined.

The clinical consequences of immunosenescence include increased susceptibility to infectious diseases, neoplasias and autoimmune diseases (Castle, 2000). However, this altered morbidity is not evenly distributed and must, therefore, be influenced by other immune-modulating factors. For instance, we have recently demonstrated that healthy elderly individuals who experience chronic stress exhibited poorer non-specific T-cell function (Bauer et al., 2000). These data led us to consider that other factors may potentially contribute to the heterogeneity of these changes, including the psychological and endocrine systems. Therefore, the understanding of the interplay between the immune, endocrine and nervous systems in the elderly is of paramount importance.

Healthy ageing has been associated with several psychological and behavioural changes, including difficulty to concentrate progressive cognitive impairments and sleep disturbances (Salzman and Shader, 1978). Although individually identified, these alterations may be associated with mood disorders, including clinical depression. However, there are several discrepancies as regards to whether ageing is associated with a higher incidence of depression (Ernst and Angst, 1995). For instance, this could be related to problems in diagnosing depression and may thus explain why some studies have reported a reduced prevalence of major depression in the elderly. In addition to being associated with other medical disorders observed in the elderly (Krishnan et al., 2002), major depression is also known to dampen cell-mediated immunity (Bauer et al., 1995). Therefore, psychological factors must be considered as important risk factors for the immunosenescence.

The endocrine system also undergoes important changes during ageing. Endocrinosenescence is particularly demonstrated by a substantial decline in several hormones (e.g. growth hormone, dehydroepiandrosterone, testosterone, progesterone and aldosterone) in contrast to an increase of some adrenal hormones (Straub et al., 2000). Regarding the latter, there is some evidence suggesting that ageing is associated with significant activation of the hypothalamic–pituitary–adrenal (HPA) axis (Halbreich et al., 1984; Van Cauter et al., 1996; Deuschle et al., 1997; Seeman et al., 2001). The HPA axis performs a tight integration among the endocrine, nervous and immune systems. Its activation results in increased production of glucocorticoids (cortisol in man) and catecholamines that modulate several immune responses (Munck et al., 1984). Increased cortisol levels are also seen in demented patients (Maeda et al., 1991) or during stress (Kirschbaum et al., 1995), or during stress (Kirschbaum et al., 1995; Bauer et al., 2000). In particular, it was observed that chronically stressed elderly subjects (caregivers of demented patients) had a blunted T-cell proliferation in association with increased cortisol levels (Bauer et al., 2000). In addition, several changes observed in the elderly (such as thymic involution, osteoporosis and lymphopenia) are also seen following chronic

glucocorticoid treatment (Munck et al., 1984). Overall, these studies suggest that endocrinosenescence may be kept in close relationship with the immunosenescence.

Despite the evidence suggesting a close communication between the neuroendocrine and immune systems, there have been few studies in this field (Straub et al., 2000; Martinez-Taboada et al., 2002). We have recently demonstrated that strictly healthy ageing was associated with significant psychoneuroendocrine activation in parallel with no changes in pro-inflammatory cytokine production (Luz et al., 2003). In this study, we further describe this elderly cohort investigating whether age-related psychological and neuroendocrine alterations were implicated with changes in distribution of peripheral blood lymphocytes. In particular, we have immunophenotyped peripheral T-cell subsets and investigated the role of psychoneuroendocrine factors in regulating the expression of CD95 on lymphocyte subsets.

2. Materials and methods

2.1. Subjects

Forty-six non-institutionalised healthy elderly (31 females, 15 males), aged from 65 to 91 years (mean age 72.0 ± 8.5 years), were recruited from an existing database of 1118 socially active elderly subjects who had previously participated in research at the Institute of Geriatrics and Gerontology (PUCRS). All subjects were recruited from local community centres and registered at the Office for Social Care in Gravataí (RS). This elderly population corresponded both ethically and socio-economically to the general population of our state (RS). All subjects took part in the GENESIS Program for the study on the genetic–environmental interactions on human ageing. Thirty-three healthy young adults (18 females, 15 males), aged from 20 to 40 years (mean age 27.4 ± 6.7 years), also took part in this study and were all students or employees from the PUCRS. The female/male ratio did not differ significantly between elderly and young subjects, $\chi^2 = 1.35$, $P = 0.25$. Most elderly (95.6%) and young subjects (81.0%) were Caucasian, $\chi^2 = 1.91$, $P = 0.17$. There were no differences regarding smoking habits between groups, $\chi^2 = 5.51$, $P = 0.14$.

All subjects were recruited accordingly to the SENIEUR protocol (Litghart et al., 1984) that defines rigorous criteria for selecting healthy individuals in immunogerontological studies. The health conditions were checked accordingly to accurate clinical investigations and to haematological and biochemical parameters. The exclusion criteria included: infections, acute or chronic inflammation, autoimmune diseases, heart disease, under nourishment, anaemia, leucopenia, clinical depression, caregiving, neurodegenerative disease, neoplasias and use of hormones (glucocorticoids) and drugs (alcohol, antidepressants, immunosuppressants, anticoagulants). All subjects had normal plasma levels of ferritin, folic acid and Vitamin B12 (data not shown),

confirming there was no nutritional changes that would influence the immunological assessments.

2.2. Experimental protocol

Subjects reported to the laboratory between 07:00 and 08:00 h and were promptly examined by a geriatrician. After the clinical examination, subjects were asked to collect the first saliva sample (9 a.m.) and blood was immediately drawn for the immune measures. Psychological assessments were performed by a trained investigator (below). Before leaving the laboratory, subjects were asked to collect the second saliva sample (12 p.m.) and were instructed to collect the third sample (10 p.m.) at home. The latter was kept in the fridge and returned to lab within a week. The study protocol was approved by the ethics committee (Pontifical Catholic University of Rio Grande do Sul, PUCRS, Porto Alegre, Brazil) and written informed consent was obtained from all subjects.

2.3. Psychological evaluation

The psychological status was assessed by means of structured clinical interviews and performed by a trained investigator (Luz et al., 2003). Depression was evaluated by a Geriatric Depression Scale (Yesavage et al., 1982) with cut-off point higher than five for the presence of depression symptoms (Shua-Haim et al., 2001). Anxiety was assessed by the Hamilton Anxiety Scale (HAM-A) with cut-off point higher than 20 for the presence of anxiety (Hamilton, 1967). Moreover, symptoms of stress were monitored by the “Inventory of Stress Symptoms for Adults” (ISSL) (Lipp and Guevara, 1994). This scale includes a quadriphasic model for the study of stress that was based on Selye’s model of stress (Selye, 1936). The Kuder–Richardson reliability coefficients for these scales were higher than 0.90. The ISSL is composed of four sections corresponding to the following stress phases: alarm, adaptation, quasi exhaustion and exhaustion. The symptoms listed in the scale are specific for each phase of stress. The scoring was performed by means of three different stages related to the duration ($Q1$ = last 24 h, $Q2$ = last week and $Q3$ = last month) and intensity of stress symptoms. The sum of all physical as well as psychological symptoms within each stage resulted in a score that is related to the presence of stress accordingly to the following criteria: $Q1 > 6$ or $Q2 > 3$ or $Q3 > 8$.

2.4. Collection of salivary samples and cortisol measurements

Salivary cortisol levels were assessed as an objective marker of the HPA axis function (Kirschbaum et al., 1995; Luz et al., 2003). Participants were asked to collect three saliva samples with the help of cotton rolls over the course of the experimental day at 9 a.m., 12 p.m. and 10 p.m. Upon arrival in the laboratory, the samples were centrifuged and

frozen at -20°C . Salivary cortisol samples were then analysed by radioimmunoassays (RIA, DPC Medlab, São Paulo, Brazil). The sensitivity of these assays was estimated in 0.1 nM/l. The intra- and inter-assay coefficients of variation were less than 10%. Results from each of the sampling times were expressed in nM/l.

2.5. Collection of peripheral blood and haematological analyses

Two millilitres of peripheral blood was collected by venepuncture in the morning (between 9 and 10 a.m.) and samples stored into lithium vacutainers prior to analyses. All samples were analysed within 4 h after collection. Full blood counts were requested to further check the health status of the subjects (e.g., leukocytosis, leucopenia, anaemia, etc.). One ml of peripheral blood was sent to haematology laboratories at Hospital São Lucas and samples were analysed within 4 h after collection using automated standard FACS analysis.

2.6. Immunophenotyping

Red cells were lysed by adding NH_4Cl (14:1) for 6 min at room temperature. The cells were then washed ($2\times$, Hanks, Sigma), resuspended in phosphate buffered saline (PBS). For two-colour staining, fluorescein (FITC) labelled monoclonal antibody and phycoerythrin (PE) labelled antibody were added to 12 mm \times 75 mm test tubes. Aliquots of the cell preparations were added to each tube. This mixture was then incubated in the dark in an ice bath for 30 min. After incubation, cells were washed with cold PBS and fixed by the adding 1% formaldehyde. All cells were stained and fixed within 4 h. The samples were stored in the dark in an ice bath, and promptly analysed using a FACScalibur (BD Pharmingen, San Diego, USA). A minimum of 10,000 lymphocytes, gated by size (FSC) and granularity (SSC), were analysed using the CellQuest software (BD Pharmingen). The cut-off for positive fluorescence was set to include $< 1\%$ of negative control mouse IgG conjugates (BD Pharmingen). Compensation levels were adjusted with two paired mAbs and checked regularly. Absolute counts for the lymphocyte subsets were calculated from lymphocyte counts (i.e. full blood counts) and the proportion of antibody positive events from the FACS analysis.

Two-colour enumeration of peripheral lymphocyte subsets were performed using the following panel of labelled monoclonal antibodies (all from BD Pharmingen): isotype controls (IgG1-FITC and IgG1-PE), anti-CD45RA-FITC, anti-CD45RO-FITC, anti-CD95-PE (Fas), anti-CD3-FITC, anti-CD4-PE, anti-CD8-PE and anti CD62L-FITC (L-selectin).

2.7. Statistical analysis

All variables were tested for normality of distribution by means of the Kolmogorov–Smirnov test. Cortisol data

were log transformed to normalise the distribution. The independence of classification systems was checked through analysis of contingency (χ^2 test). Differences between variables were assessed by independent *T* tests (psychological data) and multivariate analysis of variance (MANOVA; immune data). Differences between psychological scores across various age groups were assessed by ANOVA. The cortisol levels were analysed as two (group) \times three (time points) repeated measures ANOVA. Multiple comparisons among group mean differences were checked with Bonferroni post hoc test. All the variables that were found to be statistically associated with immunological indexes were evaluated by stepwise multiple regression analysis ($P < 0.05$ to enter in the model) in order to assess their independent relationships. Collinearity diagnostics were performed with all the dependent variables entered in the model. Thus, the predictors analysed were not confounded in this study. The significance level was set at $\alpha = 0.05$ (two-tailed) and a computer statistics package (SPSS 11.0, USA) was used for statistical analyses in this study. Results are expressed as mean \pm S.E.M. in all figures and tables.

3. Results

3.1. Ageing is associated with significant distress and activation of the HPA axis

We have previously demonstrated that this healthy elderly population was significantly distressed (Luz et al., 2003). In particular, it was observed that elders were more depressed ($P = 0.005$), anxious ($P = 0.001$) and stressed ($P < 0.001$) than young adults (Table 1). In parallel to psychological distressed, significant changes in salivary cortisol levels were also noted, $F(2, 104.88) = 155.04$, $P < 0.0001$. Post hoc analyses revealed that cortisol levels different significantly between the three sampling times, $P < 0.0001$. Cortisol peaked in the morning and presented a nadir at night, with a regular circadian pattern for both groups. Interestingly, cortisol levels were significantly elevated at all time points evaluated in the elderly compared to young adults $F(1, 71) = 9.06$, $P = 0.004$ (Table 2).

Table 1
Psychological assessments

Variables	Young ($n = 33$)	Elderly ($n = 46$)
Stress ($Q1$)	2.10 \pm 0.32	4.09 \pm 0.37****
Stress ($Q2$)	2.65 \pm 0.50	4.98 \pm 0.41***
Stress ($Q3$)	3.77 \pm 0.67	6.22 \pm 0.63**
Anxiety	3.29 \pm 0.59	5.00 \pm 0.35***
Depression	17.94 \pm 2.69	29.40 \pm 1.87**

Stress scoring ($Q1 =$ last 24 h, $Q2 =$ last week and $Q3 =$ last month).

** $P < 0.01$ vs. young subjects.

*** $P < 0.001$ vs. young subjects.

**** $P < 0.0001$ vs. young subjects.

Table 2
Salivary cortisol levels

Free cortisol (h)	Young ($n = 33$)	Elderly ($n = 46$)
9	9.93 \pm 1.29	13.82 \pm 1.56*
12	5.75 \pm 0.86	7.95 \pm 0.87*
22	2.26 \pm 0.42	4.45 \pm 0.48*

* $P < 0.05$ vs. young subjects.

3.2. Changes in peripheral blood lymphocyte subsets

Haematological analyses revealed no changes between healthy elderly and young adults (Table 3). Freshly isolated whole-blood lymphocytes were then immunophenotyped for some cogent T-cell markers. Fig. 1 shows representative FACS profiles of double marked cells. MANOVA revealed that the absolute numbers of CD3+CD4+ and CD3+CD8+ cells did not differ between elderly and young subjects (Fig. 2). However, we observed that the elderly had significant lower counts of peripheral CD45RA+CD95+ cells compared to young subjects ($P < 0.01$).

In addition, the mean fluorescence intensity (MFI) that is proportional to receptor surface density was measured from the above determinations. It was observed a reduced expression of CD3 ($P = 0.02$) and CD62L ($P < 0.05$) in lymphocytes from elderly compared to young adults (Fig. 3). In contrast, there was a trend for reduced CD8 expression ($P = 0.08$) and higher expression of CD45RO ($P = 0.05$) in lymphocytes of the elderly. We then evaluated the expression of CD95 on CD45RA/RO+ cells. In particular, it was observed that only CD45RA+ cells of the elderly presented an increased expression of Fas ($\sim 44\%$) compared to young subjects ($P = 0.0007$) (Fig. 3).

3.3. Psychoneuroimmunologic interactions during healthy ageing

We explored the complex relationships of the psychoneuroendocrine and immunological variables in this study. To explore what characteristics were predictive of immunological variables that were found to differ between young and elderly subjects, stepwise multiple regression analyses were conducted using the age, depression, anxiety, stress and

Table 3
The absolute counts ($\times 10^3 \mu\text{l}^{-1} \pm$ S.E.) of different types of peripheral blood cells

Cells	Young	Elderly
Red blood cells	4747.73 \pm 91.03	4640.44 \pm 55.52
Platelets	253.67 \pm 15.86	210.00 \pm 10.76
Leukocytes	6.60 \pm 0.42	6.79 \pm 0.20
Neutrophils	3.95 \pm 0.24	3.84 \pm 0.15
Monocytes	0.55 \pm 0.04	0.58 \pm 0.03
Lymphocytes	2.33 \pm 0.13	2.04 \pm 0.09
Eosinophils	0.17 \pm 0.03	0.27 \pm 0.04
Basophils	0.03 \pm 0.00	0.03 \pm 0.00.26

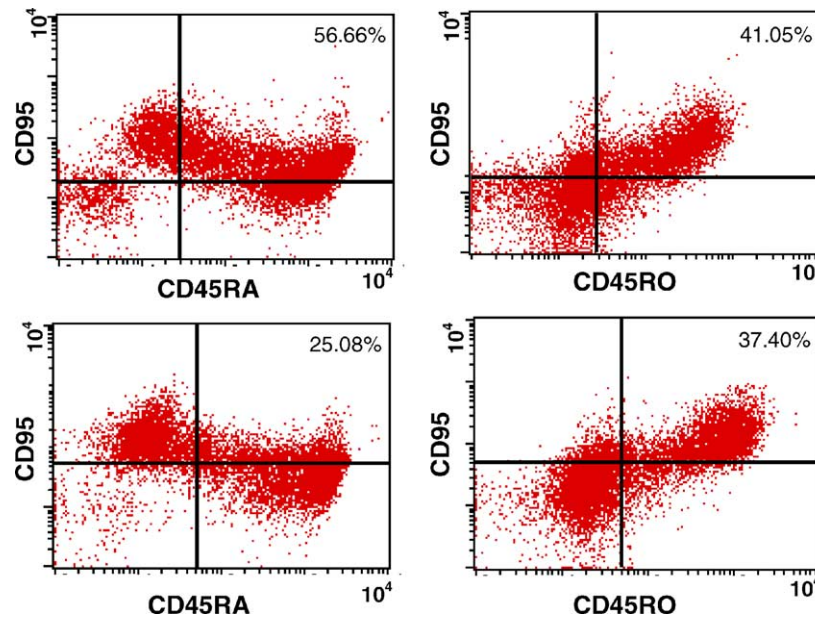


Fig. 1. Representative FACS profiles. This figure shows the expression of CD95 on both CD45RA+ and CD45RO+ cells from male young (25 years, top) and elderly (68 years, bottom) subjects.

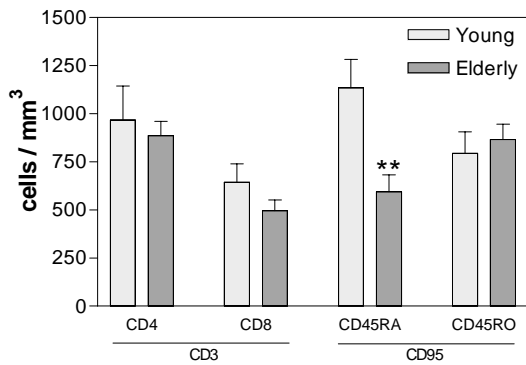


Fig. 2. Absolute counts of T-cell subsets. This figure shows the absolute counts of T helper cells (CD3+CD4+), T cytotoxic (CD3+CD8+) cells as well as co-expression of CD95 (Fas) in CD45RA+ and CD45RO+ cells. Statistically significant differences are indicated: ** $P < 0.01$ vs. young. Data are presented as mean \pm S.E.

cortisol levels as predictors. Age remained inversely associated to absolute CD45RA+CD95+ counts and expression of CD3 and CD62L (Table 4). Stress was found positively associated to CD95 expression on CD45RA+ cells. Inter-

estingly, age and cortisol (12 and 22 h) variables contributed up to 63% of the variation observed in CD62L expression. Collinearity diagnostics were performed with all the dependent variables. The condition index reported by each specific test was always lower than 7. Thus, the predictors analysed were not confounded in this study. Overall, these data suggest that both psychological and endocrine factors maybe implicated with the immunosenescence.

4. Discussion

In this study, we explored the complex interplay of immune, endocrine and nervous systems between healthy elderly and young subjects. In particular, we investigated the role of psychoneuroendocrine factors in regulating the distribution of peripheral T-cell subsets. We observed that strictly healthy (SENIEUR) elderly were significantly more distressed than young adults. In particular, the healthy elderly were more depressed, anxious and stressed than young subjects (Luz et al., 2003). This psychological distress was associated with higher salivary cortisol levels, indicating

Table 4
Stepwise multiple regression for immunological variables

Dependent variables	Predictors	Standardised coefficients (β)	Significance	ANOVA (F); R^2 (%)
CD45RA+CD95+ (n)	Age	-0.34	0.04	$F(1, 33) = 4.29, P < 0.05; 11.80$
CD3 (MFI)	Age	-0.42	0.01	$F(1, 35) = 7.50, P = 0.01; 18.10$
CD45RA+CD95+ (MFI)	Stress	0.58	<0.0001	$F(1, 37) = 18.26, P < 0.0001; 33.70$
CD62L (MFI)	Age	-0.66	<0.0001	$F(3, 32) = 16.72, P < 0.0001; 63.40$
	Cortisol (12 h)	-0.70	<0.0001	
	Cortisol (22 h)	-0.59	<0.0001	

R^2 is the percentage of explained variance. Only significant predictors are given. MFI: mean fluorescence intensity, n : counts.

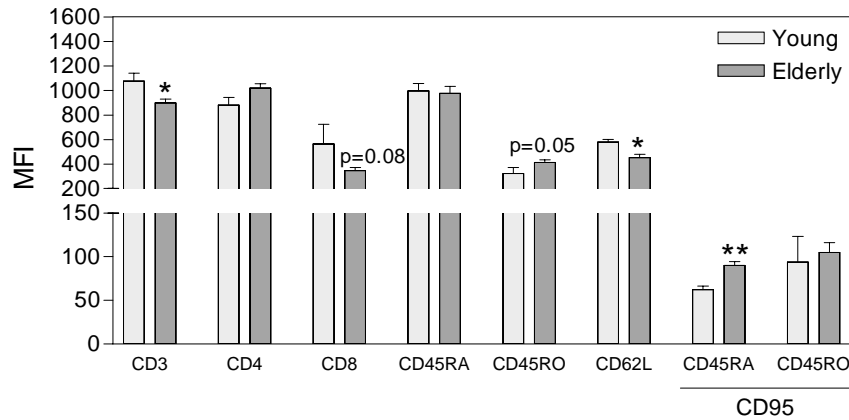


Fig. 3. Expression of T-cell markers. Data presented as mean fluorescence intensity (MFI \pm S.E.) that is proportional to receptor surface density. Statistically significant differences are indicated: * $P < 0.05$ and ** $P < 0.001$ vs. young.

activation of the HPA axis (Luz et al., 2003). Confirming previous reports, changes in lymphocyte distribution were noted in the elderly as demonstrated by a significant drop in CD45RA+ cells associated with higher expression of CD95 (APO1/FAS) in this subset. In contrast, we also observed a reduced expression of accessory molecules in lymphocytes of the elderly when compared to young controls. We demonstrated here that both psychological and neuroendocrine (cortisol) factors were correlated in those alterations that are known to characterise the immunosenescence process. Although correlation does not prove causal relationship, it indicates that this line of evidence should merit further investigation.

Ageing is associated with several immune-related diseases including cardiovascular, infectious and cancer. Therefore, to control for age-related diseases that would interfere with our immunological analyses, strictly healthy individuals were recruited by means of the SENIEUR protocol (Litghart et al., 1984). However, we observed that SENIEUR elderly subjects had elevated BMI compared to young adults. Changes in BMI could be related to both obesity and lack of regular exercise that have been implicated to immunological changes (Pedersen and Toft, 2000; Lamas et al., 2002). However, our elderly cohort did not seem to have nutritional changes as they reported significantly higher serum ferritin and folic acid levels compared to young adults (submitted for publication).

In this study, we demonstrated that healthy ageing is associated with significant psychological changes. It is noteworthy to address here, however, that the elderly subjects investigated in this study were not suffering from clinical depression or chronic stress. In fact, all were non-institutionalised and socially active individuals. The literature regarding age-related psychological changes is controversial and others did not find these changes (Nolen-Hoeksema and Ahrens, 2002). This could be due to methodological issues, since specific clinical interviews are required to assess depression in the elderly. For instance, most elderly subjects do not clearly declare symptoms of

sadness and tiredness and are more inclined to pretend their feelings in order to act more self defensibly towards questions that may threaten their self esteem (Snowdon, 2001).

In parallel to psychological changes, we observed that healthy elderly subjects had increased salivary cortisol levels throughout the day. These data support previous studies (Ferrari et al., 2001) and suggest that human ageing is associated with significant activation of the HPA axis. This particular change could be due to some defects in central regulatory pathways of the axis, including a neuronal loss, especially affecting both the hypothalamus and hippocampus, and implies a reduction in GC receptors and hence an impaired regulation of the adrenocortical secretion (Mani et al., 1986). Consequently, the degeneration of the hippocampus may be responsible for a decreased sensitivity to cortisol and hyperactivation of the HPA axis during ageing (Sapolsky et al., 1986). However, there are studies in the literature reporting that cortisol levels are unchanged during healthy ageing (Waltman et al., 1991). These discrepancies could be related to small sample sizes, heterogeneity of subject populations in terms of gender distribution, age range, healthy status, differences in the analytical methods employed and psychological status. As regards the latter, there is evidence suggesting the stress-related HPA axis activation may produce the very same neurological changes in the limbic region via the direct action of cortisol in these areas. Therefore, it becomes difficult to dissociate these neuroendocrine changes observed in the elderly with those induced by psychological stimuli. For instance, we have previously demonstrated that chronically stressed elderly subjects had increased salivary cortisol levels compared to non-stressed elderly (Bauer et al., 2000). In this study, psychological scores were positively correlated to cortisol levels (Luz et al., 2003). These results are in line with previous studies that observed an activation of the HPA axis during depression (Stokes, 1995; Lupien et al., 1999). Taken together, these data suggest that psychological factors may be implicated in the HPA axis activation during healthy ageing.

Moreover, we also described here age-related changes in T-cell subsets. In accordance to previous studies (Hannet et al., 1992; Ginaldi et al., 2000; Martinez-Taboada et al., 2002; Peres et al., 2003), we observed that ageing was associated with a significant drop in CD45RA⁺ cells. This could be due to age-related thymic involution (Aspinall and Andrew, 2000), leading to a reduced production of naive T cells and consequently accumulation of memory T cells. However, our findings should be interpreted with caution because CD45RA/RO markers are also expressed by other cells including effector T cells (Höflich et al., 1998), NK T cells (Van Der Vliet et al., 2000), B cells and monocytes (Barclay et al., 1993). There is also some evidence that NK cells from healthy adults strongly expressed CD45RA^{bright} and weakly expressed CD45RO (Van Der Vliet et al., 2000). Although some studies reported an age-related increase in NK cells (Ginaldi et al., 1999; Malaguamera et al., 2001), it remains to be investigated whether NK⁺ CD45RA^{bright} cells are also increased during ageing. However, considering that we gated on peripheral lymphocytes in which only a small proportion are B cells (~5–10%), NK or NK T cells (~5%), we can assume that most cells stained for CD45RA are in fact T cells. Further studies should perform three- or four-colour flow cytometric analyses in order to circumvent this problem. A drop in CD45RA⁺ cells was also observed in strictly selected elderly individuals, occurred independently of health or nutritional status (Mazari and Lesourd, 1998), and may be associated with a poor cell-mediated immunity observed in the elderly. In mice, for example, the accumulation of memory T cells was associated with a reduced mitogen-induced lymphocyte proliferation (Lerner et al., 1989) and may thus possibly contribute to a poor *in vivo* immune response against new antigens.

The mechanisms underlying the regulation of the peripheral pool of lymphocytes are still largely unknown. It has been speculated that CD95 (APO1/FAS) may be involved in this process through engagement of apoptosis. In this study, we observed that ageing was associated with increased expression of CD95 in CD45RA⁺ cells only. This differential expression of CD95 may potentially select “putative naive T cells” for apoptosis and could explain peripheral reductions in CD45RA⁺ cells. In contrast, another study observed that ageing was associated with a reduced expression of CD95 in CD4⁺ CD45RA⁺, CD8⁺ CD45RA⁺ and CD8⁺ CD45RO⁺ cells, but not in CD4⁺ CD45RO⁺ cells, suggesting that memory T helper cells may be resistant to apoptosis (Aspinall et al., 1998). The increased expression of CD95 in CD45RA⁺ cells may be a compensatory mechanism related to lower counts of these cells during ageing. However, current evidence is still controversial. Some studies reported an age-related decrease in expression of CD95 on CD45RO⁺ cells (Herndon et al., 1997) while others found that ageing increased the expression of CD95 on both CD45RO⁻ and CD45RO⁺ lymphocytes (Potestio et al., 1999). As CD95 (Fas/APO-1) expression on T cells is a marker for previous antigenic contact (Höflich et al., 1998),

the increased CD95 expression in CD45RA⁺ cells may also be due to increased effector cell numbers previously observed during human ageing (Zanni et al., 2003). Overall, these results indicate that ageing may alter lymphocyte's sensitivity to apoptosis through expression of CD95.

Glucocorticoids may also contribute to the immunophenotypical changes observed during ageing. It has been demonstrated that glucocorticoid-induced apoptosis on monocytes is at least partially mediated by the expression of both CD95 and CD95L (Schmidt et al., 2001). Another study showed that glucocorticoids may either induce T-cell apoptosis in a CD95-independent manner, or protect T cells from CD95-mediated apoptosis (Zipp et al., 2000). Furthermore, there is some evidence that psychological stress may regulate the proportion of peripheral lymphocytes via the expression of CD95. For instance, it has been demonstrated that chronic stress may induce lymphocyte apoptosis in mice (Yin et al., 2000) or in man (Oka et al., 1996) via upregulation of CD95. Our results support the concept that age- or stress-related increase in cortisol levels may be preferentially altering the expression of CD95 on CD45RA⁺ cells. Preliminary data from our laboratory indicate that human CD45RA⁺ CD95⁺ cells are in fact more sensitive to dexamethasone treatment *in vitro* (unpublished results).

The HPA axis is pivotal for the homeostasis of the immune system and its dysregulation has been associated with several immune-mediated diseases (McEwen et al., 1997). Thus, chronic HPA axis over-activation, as occurs during stress, can affect susceptibility to or severity of infectious disease through the immunosuppressive effect of the glucocorticoids (Glaser et al., 1994; Vedhara et al., 1999). In contrast, blunted HPA axis responses are associated with enhanced susceptibility to autoimmune inflammatory disease (Sternberg, 2002). It is noteworthy to mention that elderly subjects are particularly at risk for both infectious and chronic inflammatory diseases. Therefore, dysregulation of the HPA axis may thus contribute to human immunosenescence. We observed in this study that psychological factors were positively correlated to several peripheral T-cell markers. These data are in line with previous studies that found stress-related changes in CD62L expression (Adler et al., 2002). These data suggest that stress may be associated with changes in cellular distribution, such as redirection of lymphocytes to peripheral lymph nodes—since CD62L is necessary for lymphocyte homing to these secondary lymphoid organs. Chronic stress (Adler et al., 2002), depression (Bauer et al., 2002) and anxiety (Manfro et al., 2000) have consistently been associated with changes in cellular trafficking—in young as well as in elderly populations. Other studies also demonstrated that depression and anxiety were related to significantly increase in CD45RA⁺ cells and reduced CD62L expression (Manfro et al., 2000). Data produced in this study are partially supported by these findings. Although both autonomic nervous system and HPA axis have been implicated with these changes, increased levels of glucocorticoids are critically involved with these

changes. In addition, GC treatment *in vivo* has also been associated with similar changes in cell trafficking (Bauer et al., 2002), including lymphopenia, neutrophilia and reduced CD4/CD8 ratio. These changes have also been ascribed to immunosenescence. Therefore, changes produced by emotional distress, GC treatment and ageing are indeed very similar. However, our data cannot be directly compared with previous studies because (a) the elderly subjects investigated in this study were not suffering from clinical depression or chronic stress and (b) previous studies have not assessed the impact of emotional variables on peripheral T cell subsets of SENIEUR elders. We believe the emotional alterations reported here are inherent of healthy ageing and there are probably no “happy” healthy aged individuals to be compared to.

In summary, we demonstrated in this study that healthy ageing is associated with significant psychological, endocrine and immunological changes. Furthermore, we suggested that there are complex psychoneuroendocrine interactions involved with the regulation of the peripheral pool of lymphocytes. Additional research is necessary to further explore these interactions in both healthy and pathological ageing. A better understanding of the physiological interplay of the immune, endocrine and nervous systems may help to define new therapeutic strategies to modulate the immunosenescence and promote the quality of health during ageing.

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