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Communication

Does *in vitro* replicative senescence of human CD8$^+$ cells reflect the phenotypic changes observed during *in vivo* ageing?

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Immunosenescence is viewed as a remodeling process with the exhaustion of naïve T cells and filling up of the immunological space with memory cells. In this study some phenotypic changes of CD8$^+$ human cells during *in vivo* ageing were compared with those observed in long term cultures of lymphocytes derived from cord blood or from peripheral blood from donors of different age. Both *in vivo* and *in vitro* a significant decrease of the fraction of CD8$^+$CD28$^+$ cells was observed. Comparing the proportions of other T cell subpopulations (the CD4/CD8$^+$ ratio, CD56, CD57, CD27) made it possible to conclude that replicative senescence *in vitro* partially reflects *in vivo* ageing.

Keywords: ageing, lymphocyte subpopulations, CD8, CD28, proliferation, *in vitro* culture

Immunosenescence is a complex remodeling process during which some parameters decrease, some increase, and others remain unchanged (the remodeling theory of ageing) (Franceschi *et al.*, 2000b). Ageing of the immune system is described as exhaustion of naïve cells pool due to thymic involution and filling up of the immunological space by memory and effector T cell clones as a consequence of chronic antigenic stress (Franceschi *et al.*, 2000a). A large body of evidence indicates that after stimulation by antigens CD8$^+$ cells are induced to proliferate, undergo clonal expansion and change their phenotypic and functional characteristics. This process is described as maturation or differentiation (Appay & Rowland-Jones, 2004; Papagno *et al.*, 2004). The replicative exhaustion of lymphocytes and a decrease of immune function occur, at least partially, as a result of differentiation (Papagno *et al.*, 2004). Lymphocytes isolated from whole blood and cultured *in vitro* undergo replicative senescence, which is believed reflect to some extent *in vivo* ageing after antigenic stimulation. However, so far there is no data about the phenotypic changes of lymphocytes in long term cultures *in vitro*. Thus, this study was undertaken to compare the phenotypic changes of human T lymphocytes which occur during *in vivo* ageing with those appearing during replicative senescence *in vitro*.

MATERIALS AND METHODS

Cell isolation. Peripheral blood was collected from healthy young (25–35 years, group II) and middle-aged (45–55 years, group III) volunteers, as well as from elderly ones (65 years, group IV) and centenarians (taking part in the Polish Centenarian Project, group V). Lymphocytes were also isolated from cord blood (group I). The number of donors tested in each experiment is indicated in figure legend. Mononuclear cells were prepared by standard centrifugation over Ficoll-Paque.

Lymphocytes isolated from cord blood and from peripheral blood of donors from II and III age groups were cultured *in vitro* for 30–35 days in the presence of IL-2. Lymphocyte activation was induced with lectin PHA at 5 μg/ml (Sigma) added at the time of seeding (day 0). All cell cultures were conducted in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and antibiotics, at a starting density of 0.8×10^6 cells/ml. From day 3, cells were washed with PBS and 60 U/ml of recombinant IL-2 (rIL-2; Peprotech EC Ltd, UK) with fresh medium added.

Cell phenotyping. Cells from blood of all subjects were phenotyped on a flow cytometer using Simultest and other sets of antibodies from Beckton-Dickinson (anti-CD27-FITC, anti-CD56-FITC,

Abbreviations: CBL, cord blood lymphocytes; FCS, fetal calf serum; NK, natural killer; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin.

anti-CD57-FITC, anti-CD8-PerCP, anti-CD28-PE). To define single subsets of $CD28^+$ and $CD28^-$ among $CD8^+$ cells we used the gate of viable cells uniquely identifying $CD8^{high}$ T cells to exclude $CD3^-CD8^+$ NK cells. Samples were recorded and analyzed on a FACS Calibur (Beckton-Dickinson, Warsaw, Poland) using the Cell-Quest software (Beckton-Dickinson).

Statistical analysis. Statistical analysis of data were performed using Statistica 6.0. Non-parametric tests were applied. Parameters between age groups were compared with Kruskal-Wallis tests for paired observations. All results are given as mean ±S.E.M. P values < 0.05 were considered statistically significant, $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$.

RESULTS AND DISCUSSION

During ageing continuous phenotypic changes of T cell are observed. The best documented changes concern an increase of memory and a decrease of naïve cells as well as a dramatic drop of $CD8^+$ T cells with the $CD28^+$ co-receptor and concomitant accumulation of $CD8^+CD28^-$. In *in vitro* replicative senescence a very well known fact is an increase in the proportion of $CD8^+CD28^-$ cells with diminished proliferation capacity. However, so far there is no data comparing other phenotypic parameters occurring during ageing *in vivo* and senescence *in vitro*. Here, I present data showing lymphocyte subpopulation changes during *in vivo* and *in vitro* senescence.

The CD4 and CD8 subpopulations in *in vivo* and *in vitro* ageing

The absolute number and the percentage of CD4 and CD8 lymphocytes in peripheral blood, as well as the CD4/CD8 ratio, seem to be an important parameter of health status. The lower CD4/CD8 ratio, together with some other immune parameters predicts a higher risk of death (Pawelec *et al.*, 2004). However, there are contradictory reports showing either a decrease in absolute number of $CD4^+$ and increase of $CD8^+$ cells (Wikby *et al.*, 2002; Huppert *et al.*, 2003), decrease of $CD8^+$ (Naumova *et al.*, 2004), or no changes in either parameter during ageing (Peres *et al.*, 2003). In this study lower mean values of $CD8^+$ than of $CD4^+$ cells were observed in donors from groups I–V. The CD4/CD8 ratio in peripheral blood of all donors ranged between 1.8–2, being significantly greater in cord blood (3.0). The percentage of centenarian donors with CD4/CD8 ratio below 1 was higher (25%) than in group IV (5%) (Fig. 1A). There was only a single donor with the CD4/CD8 ratio < 1 in group III. Thus, the older the age-group, the more donors exhibited a relative domination of the CD8 subpopulation, which might reflect the previously described age-related immunodeterioration (Pawelec *et al.*, 2004). In *in vitro* culture of PBL and CBL a predominance of $CD4^+$ cells was observed at the beginning of culture; after 7th day of PBL culture and after 14th day of CBL culture, $CD8^+$ cells prevailed. This picture persisted until the end of CBL culture (about 35th day of culture). In PBL culture, the CD4/CD8 ratio changed again at the end of culture (Fig. 1B, C). These results suggest that in PBL culture $CD8^+$ cells divided slower or underwent less division rounds, comparing to $CD4^+$ cells. A similar observation of $CD4^+$ lymphocyte expansion and a decrease of $CD8^+$ cells *in vitro* was made by Dennett *et al.* (2002). Contrary to this, an expansion of $CD8^+$ population was observed in culture with continuous restimulation (Spaulding *et al.*, 1999). Thus, $CD8^+$ cells cultured *in vitro*, in contrast to $CD4^+$ cells, need to be repeatedly stimulated with exogenous antigen to maintain high proliferative activity.

The $CD8^+CD28^+$ and $CD8^+CD28^-$ subpopulations in *in vivo* and *in vitro* ageing

The best documented hallmark of immunosenescence is a reduction of the relative amount of $CD8^+$ lymphocytes with CD28 expression (Nociari *et al.*, 1999; Posnett *et al.*, 1999). In this study, we also showed on the large number of donors the age-dependent decrease of $CD8^+CD28^+$ subpopulation, with concomitant increase of $CD8^+CD28^-$ subpopulation. The percentages of $CD8^+CD28^+$ cells were 75, 61, 55 and 35% in II, III, IV and V age-groups, respectively (Fig. 2A). In CBL, 100% of the $CD3^+CD8^+$ subpopulation were $CD28^-$ positive.

Similar changes in $CD8^+CD28^+$ and $CD8^+CD28^-$ proportions were observed during *in vitro* ageing. The percentage of the $CD8^+CD28^+$ subpopulation gradually decreased with time. The decrease of the $CD8^+CD28^+$ subpopulation was faster in CBL than in PBL cultures; a dramatic drop from almost 100% of $CD8^+CD28^+$ cells at the beginning of culture to 40% after two weeks was observed. However, at the end of culture irrespective of the age of donor some cells still displayed the $CD28^+$ phenotype and the average percentage of $CD8^+CD28^+$ cells at the 35th day of culture was 12, 35, 42 and 20% in age groups I, II, III and V, respectively (Fig. 2B).

In all donors tested I observed decrease of the number of $CD8^+CD28^+$ and a concomitant increase of the number of $CD8^+CD28^-$ cells both *in vivo* and *in vitro*, thus proving that *in vitro* replicative senescence reflects — at least considering this parameter — the ageing process occurring *in vivo.*

The question is whether the $CD8^+CD28^-$ subpopulation is the progeny of $CD8^+CD28^+$ cells. Actually, there are some data showing that $CD28^-$ cells appear as progeny of $CD28^+$ cells, after down-regulation of CD28 receptor expression (Fiorentini *et al.*, 1999; Borthwick *et al.*, 2000). In cultures of isolated

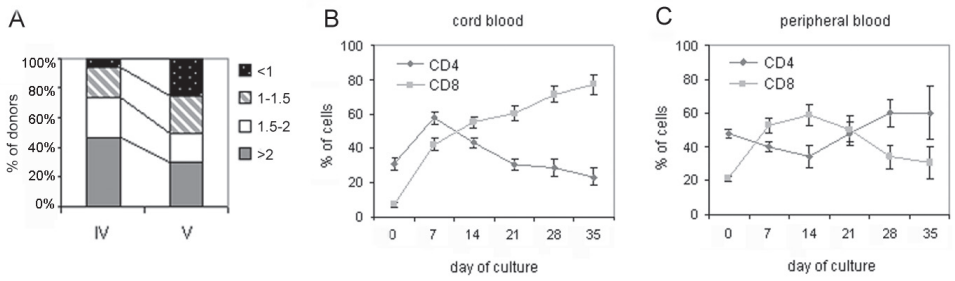

Figure 1. The CD4⁺/CD8⁺ subpopulation ratio in *in vivo* ageing (A) and *in vitro* culture (B, C).
A. Percentage of donors displaying different CD4/CD8 ratios (< 1, 1–1.5, 1.5–2, >2) in group IV (elderly and V, centenarians). B, C. Percentage of $CD4^+$ and $CD8^+$ subpopulations isolated from cord blood (B) and peripheral blood (C) during *in vitro* culture. Results were obtained from seven CBL and nine PBL cultures from donors from groups II and III.

fractions or clones of $CD8^+CD28^+$ cells, $CD28^-$ cells have been reported to appear (Fiorentini *et al.,* 1999; Posnett *et al.,* 1999; Borthwick *et al.,* 2000). Moreover, in $CD28^+$ and $CD28^-$ subpopulations the same TCRVβ clones were expanded (Horiuchi *et al.,* 2001). On the other hand, there are data about an extrathymic lineage of these cells (Tsukahara *et al.,* 1997). Azuma *et al.* (1993) showed $CD8^+$ clones with the $CD28^+$ phenotype lasting for 4 weeks in culture, arguing that the loss of CD28 expression contributed insignificantly to the appearance of $CD28^-$ cells. The results presented in this study show that CBL, being virtually all the $CD8^+CD28^+$, are gradually replaced with $CD8^+CD28^-$ cells. In CBL the decrease of $CD8^+CD28^+$ percentage was more intense (even 80% during four weeks of *in vitro* culture, Fig. 2B) and was accompanied by a higher proliferative activity of these cells comparing to PBL (not shown). This data is consistent with the conclusion that $CD28^-$ cells are the progeny of $CD28^+$ cells and appear in the process of antigen-driven differentiation. Presumably, proliferation of $CD28^+$ cells cause the loss of CD28 receptor (Posnett *et al.,* 1999; Borthwick *et al.,* 2000).

The CD27, CD56 and CD57 subpopulations in *in vivo* and *in vitro* ageing

Differentiation of lymphocytes is induced by antigenic stimulation and is marked by decreasing expression of CD28 and CD27 receptors. The following phases of $CD8^+$ T cell differentiation, based on the CD28 and CD27 phenotype, were proposed: early $CD28^+CD27^+$, intermediate $CD28^-CD27^+$ and late $CD28^-CD27^-$ (Appay & Rowland-Jones, 2004). In fact, during *in vivo* ageing a decrease of the percentage of $CD8^+$ cells expressing the CD27 receptor was observed (Fig. 3A, $P<0.001$), and the most important phenotypic changes concerned the percentage of the $CD28^+CD27^+$ and $CD28^-CD27^-$ subpopulations. The $CD8^+CD28^-CD27^-$ cells expanded significantly (up to 60.9% in centenarians, not shown). The intermediate subpopulation ($CD28^-CD27^+$) constituted almost the same percentage of the $CD8^+$ pool in all age groups. Only in donors with acute infections or after transplantation was a great increase of the percentage of this subpopulation reported (Roos *et al.,* 2000; Wills *et al.,* 2002). I obtained a similar result in cultures senescing *in vitro,* in which $CD8^+$ cells were losing CD28 expression without concomitant down-regulation of CD27 expression. Until the 30th day of culture more than 90% of $CD8^+$ cells were CD27 positive (Fig. 3B). Thus, unlike during ageing *in vivo,* no relationship was found between the appearance of the $CD8^+CD28^-$ and $CD8^+CD27^-$ subpopulations *in vitro*. Similary to *in vitro* stimulation, decrease of CD28 expression on $CD8^+$ cells without CD27 down-regulation is a common feature of acute viral infection, during which multiple rounds of division in relatively short period occur (Roos *et al.,* 2000; Papagno *et al.,* 2004). This suggests, that — comparing to CD27 expression — the down-regulation of CD28 expression is more dependent on completed divisions.

The proportion of T lymphocytes bearing NK markers (CD56, CD57) increases with ageing (Tarazona *et al.,* 2000; Wikby *et al.,* 2002). In this study, a higher percentage of cells with the CD56 and CD57 receptors was observed in older donors than in

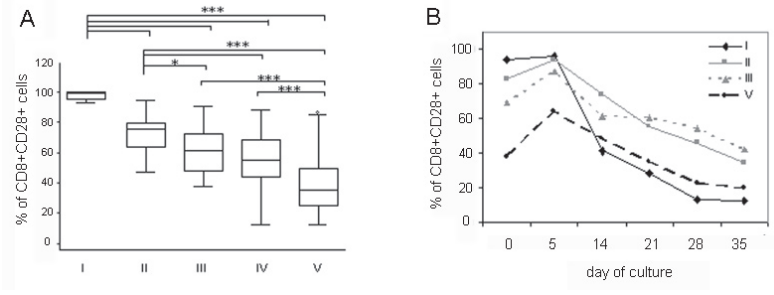

Figure 2. The percentage of $CD8^+CD28^+$ subpopulation *in vivo* (A) and *in vitro* (B).
Analyses were performed: A, in whole blood of 13, 15, 22, 57 and 108 donors from age groups I–V, respectively; B, during *in vitro* culture of lymphocytes isolated from seven donors from I group, eight donors from II group, seven donors from group III and ten from group V.

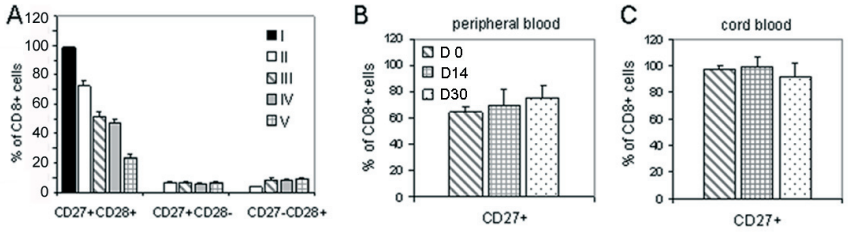

Figure 3. The percentage of $CD8^+CD27^+$ subpopulation in ageing *in vivo* (A) and *in vitro* of PBL (B) and CBL (C) cultures.
Analyses were performed: A, in the whole blood of 5, 22, 13, 48 and 47 donors from age groups I–V, respectively; B, during *in vitro* culture of lymphocytes isolated from five donors from group I and 12 donors from groups II and III.

younger ones (Fig. 4A). In CBL all cells lacked the cytotoxic markers, which confirms the 'naïve phenotype' of newborns' blood. The percentage of the $CD8^+CD57^+$ subpopulation increased during *in vivo* ageing to a higher level than of the $CD8^+CD56^+$ subpopulation. The most significant differences were observed between I and III age groups, in further life there was a less significant increase of the percentage of $CD56^+$ and $CD57^+$ cells.

Lymphocytes with NK markers have been reported to have shorter telomeres, which reflects progress in replicative senescence (Tarazona *et al.*, 2000). Acute viral infection induces high proliferation and clonal expansion in the $CD8^+$ subpopulation and the expanded cells tend to express cytotoxic markers (Looney *et al.*, 1999; Khan *et al.*, 2002). The common CMV infection is noted more frequently in older people (Khan *et al.*, 2002) and correlates with an increased number of $CD8^+CD57^+$ cells (Ouyang *et al.*, 2003). Published data suggest, that the relationship between CMV infection, $CD8^+CD57^+$ expansion and reversed CD4/CD8 ratio exist (Wikby *et al.*, 2002). In centenarians, I observed a large amount of $CD8^+CD57^+$ cells in donors with the CD4/CD8 ratio below 1, as well as with a ratio above 1. Thus, the appearance of $CD57^+$ cells is probably more dependent on age than on the CD4/CD8 ratio. Similar conclusions were offered by Merino *et al.* (1998).

During *in vitro* culture, cells isolated from CBL and PBL showed a different tendency in CD56 and CD57 expression changes, comparing to ageing *in vivo*. In PBL culture, in contrast to *in vivo* ageing, the percentage of $CD8^+CD57^+$ cells decreased gradually ($P<0.05$ in day 14 and day 30 *vs* day 0) (Fig. 4B). The $CD8^+CD56^+$ subpopulation changes were not statistically significant. Thus, in respect to acquiring the NK phenotype, $CD8^+$ lymphocyte ageing *in vivo* differs from *in vitro* replicative senescence of $CD8^+$ cells isolated from peripheral blood. However, during culture of CBL there was a significant increase of $CD8^+CD56^+$ and $CD8^+CD57^+$ percentage, similarly to changes observed during *in vivo* ageing ($P<0.05$ in day 14 and day 30 *vs* day 0) (Fig. 4C). In CBL, the NK expressing cells were formed *de novo* during culture *in vitro.* The reason of these differences is unknown.

Altogether, this study shows that some phenotypic changes are common for ageing *in vivo* and *in vitro*. The evolution of cells with the naïve phenotype into highly differentiated effector/memory cells seems to be driven, first of all, by stimulation and proliferation. An understanding of the mechanisms of lymphocyte maturation may contribute to the development of strategies protecting from age-related decline in immune functions. For this reason, *in vitro* culture of human lymphocytes is useful for studies concerning particular parameters of immunosenescence undergoing *in vivo.*

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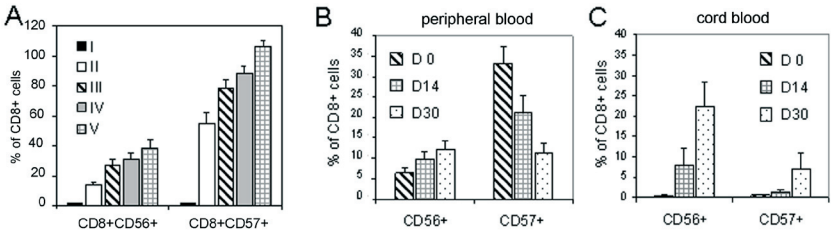

Figure 4. The percentage of $CD8^+CD56^+$ and $CD8^+CD57^+$ subpopulations in ageing *in vivo* (A) and *in vitro* of PBL (B) and CBL (C) cultures.
The analyses were done as described in the legend to Fig. 3.

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