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Immunophenotypical Changes of T Lymphocytes in the Elderly

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Key Words
Immunosenescence · T lymphocytes · Natural killer cells · Flow cytometry

Abstract
Background: Substantial changes in both representation and function of T lymphocyte subsets have been reported with advancing age. However, till now, no systematic studies focused on age-dependent changes in the expression intensity of the major T lymphocyte surface receptors. ***Objective:*** The present study was undertaken in order to establish age-related differences in lymphocyte subpopulations by simultaneously measuring three surface antigens in young and elderly people. ***Method:*** Peripheral blood T cell subsets from 20 healthy elderly individuals and 15 healthy young adult donors were examined by means of a quantitative three-color flow cytometry method. ***Results:*** Activated (HLA-DR+) and memory (CD45RO+) T cells, CD3+CD7– T lymphocytes, and cells expressing natural killer (NK) markers (CD3–CD56+ NK cells and CD3+CD56+ T lymphocytes) were expanded, whereas T lymphocytes expressing the adhesion molecule CD62L were lower in elderly compared with young donors. In addition to alterations in the percentages of T cell subsets during senescence, several changes in the intensity expression of T cell antigens were also detected. CD3 antigen expression was downregulated on total T lymphocytes as well as on the memory T cell subset, while CD56+ T cells exhibited increased CD3 levels. Moreover, CD2 expression, unchanged on NK cells, was upregulated on T lymphocytes from elderly subjects. CD3+CD7– T cells exhibited increased expression of CD8 antigen, while the intensity expression of HLA-DR on activated T cells and CD7 on both T and NK lymphocytes was decreased. T cells from elderly subjects also exhibited higher expression of CD50 and CD62L adhesion molecules as compared with young ones. ***Conclusion:*** These T cell antigen expression modulations during senescence, in addition to the alteration in the frequency of the various T lymphocyte subsets, could contribute to the complex remodeling of the immune function characteristic of the elderly.

Introduction

Several changes occur in the immune system with advancing age, probably contributing to the decreased immunoresponsiveness in the elderly [1, 2]. The main clinical manifestation of this immune dysregulation is an increased susceptibility to infections and neoplastic and autoimmune diseases, frequently observed in the elderly [3]. Although both cellular and humoral immune responses are modified with advancing age, the loss of effec-

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tive immune activity is largely due to alterations within the T cell compartment [4]. Substantial changes in both functional and phenotypic profiles of T cells have been reported with advancing age: thymic involution [4], decrease in the proportion of naive T cells with a concomitant increase in T cells with an activated/memory phenotype [1], hyporesponsiveness of T lymphocytes [5], defective natural killer cell activity [6], altered cytokine secretion [7–9], etc. The cellular and molecular bases for this age-related decline in immunocompetence are still unknown. Moreover, although many investigators have examined age-related changes in lymphocyte subsets, the results are often contradictory. A reason for these discrepancies is that the observed alterations could be a consequence of underlying pathological processes which are frequent in the elderly. For this reason a careful selection of healthy elderly people, including centenarians, who represent an example of successful aging, is a critical point in these kinds of studies. Several dysfunctions in different peripheral blood T cell subsets, affected in their frequency and/or exhibiting altered phenotypes, can contribute to the alteration of the immune function characteristic of the elderly. Such abnormalities are due to a differential subset distribution between young and old persons as well as to functional alterations of specific lymphocyte subsets. The latter could be partly due to altered production of cytokines, in addition to altered expression of functionally important molecules on the cell surface which are critical for effective lymphocyte function. These kinds of immunologic parameters could represent a useful alternative to difficult and time-consuming functional tests [10] and could provide interesting acquisitions on the immunosenescence process through the identification of further immunological characteristics of lymphocytes from elderly people. Flow cytometry is a technology whereby large numbers of cells can be analyzed and divided into functional subsets based on the expression of surface antigens. These antigens are important cellular receptors. The majority of them are commonly utilized in routine immunophenotyping, while others identify specific functional states of the analyzed cells [10]. There have been a number of published references examining lymphocyte subsets in elderly people [11–14]; however, only very recently, simultaneous analysis of three antigens has become routinely available. Reagents are in fact now available which allow simultaneous assessment of three different fluorescence [15] wavelengths on most commercially available flow cytometers.

Such three-color analyses provide more information than single- or dual-color analyses. The present study was undertaken in order to establish age-related differences in lymphocyte subpopulations by simultaneously measuring three surface antigens in young and elderly people.

Moreover, only few and/or sporadic reports consider the intensity of expression of different functional molecules on different lymphocyte subpopulations. For example, Cossarizza et al. [16] noted a clear-cut increase of high epitope density expression of the integrins on T cells, while others [1, 3, 17] described an increase of 'mature' NK cells with low CD56 expression.

Such an information could be particularly useful not only in clinical practice for the assessment of pathological conditions which are characterized by specific alterations in the number of molecules per cell (autoimmune, infectious, and oncological diseases which are increased in the elderly) [1, 3, 17], but also in the understanding of physiological changes of the immune system during senescence. In fact, the molecular and cellular basis of the remodeling of immune functions during immunosenescence could be partly responsible or be a consequence of the modification of the expression, on the cell membrane, of important receptors mediating different immunological signals. These age-dependent changes within the immune system greatly hinder any attempt to assess pathological alterations of immunological variables in elderly people. In the present study, we have focused on the age-dependent changes of T lymphocyte subsets, including the varying expression of adhesion receptors and other functional molecules. The results indicate that both the distribution of the various T cell subpopulations and the levels of expression of some surface receptors on specific lymphoid subpopulations are different between young and old individuals.

Materials and Methods

Subjects and Blood Samples

Peripheral blood from 20 healthy elderly donors (11 males and 9 females, ranging in age from 82 to 100 years, mean age 92 years) and from 15 young healthy volunteers (6 males and 9 females, ranging in age from 25 to 50 years, mean age 31 years) was collected by venipuncture into EDTA Vacutainer tubes and analyzed in order to evaluate the T lymphocyte subset distribution and the intensity of expression of the most common T cell antigens and adhesion molecules. A quantitative three-color flow cytometry method was used. All donors, from whom informed consent was previously obtained, were in good physical and mental health; the main hematological and biochemical parameters were within the normal range, and all aged donors fulfilled the admission criteria for gerontological studies proposed by the SENIEUR Protocol [18]. Blood samples were processed immediately after collection. White blood cell and differential counts were performed with a Coulter STKS hemocytometer (Coulter Electronics, Hialeah, Fla., USA).

Table 1. Monoclonal antibody combinations, fluorochromes, and source and amounts of antibody used in each staining protocol

Monoclonal antibodies	Fluoro-chromes	Volume µl	Clones
Protocol 1			
CD3	Pc5	10	UCHT1
CD7	Pe	20	3AIE-12H7
CD8	FITC	20	SFCI21THY2D3
Protocol 2			
CD3	Pc5	10	UCHT1
CD4	FITC	20	SFCI12T4D11
HLA-dr	PE	20	B8.12.2
Protocol 3			
CD3	Pc5	10	UCHT1
CD2	FITC	10	SFCI3Pt2H9
CD56	PE	20	N901
Protocol 4			
CD3	Pc5	10	UCHT1
CD45RA	FITC	10	2H4LDH11LDB9
CD45RO	PE	20	UCHL1
Protocol 5			
CD3	PC5	10	UCHT1
CD50	PE	20	HP2/19
CD62L	FITC	20	SFCI28TI766

Pc5 = PE-cyanine 5.1; FITC = fluorescein isothiocyanate; PE = phycoerythrin.

Immunostaining

Immunofluorescence staining was performed by using a standardized lysed whole-blood staining technique [16–19]. Monoclonal antibodies directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (RD), and PE-cyanine 5.1 (PC5) were combined in each tube according to five different three-color staining protocols (table 1). Directly labeled isotypic mouse immunoglobulins (MsIgG-FITC/RD/PC5) were used as a negative control in all experiments. Briefly, the appropriate saturating amounts of each monoclonal antibody, as recommended by the manufacturer, were added to 100 µl of well-mixed anticoagulant whole blood. The tubes were vortexed and then incubated on ice for 15 min. Subsequently, samples were processed for lysis and fixation by using a standardized technique (Coulter Q Prep). Since the method of cell preparation as well as the conditions under which analyses are performed may influence the level of antigen staining, to assure high reproducibility and stability of the fluorescence measurements, all our experiments were done using a well-standardized procedure with the same sample processing, staining protocols, and instrument settings.

Flow Cytometry Analysis

Flow cytometry was performed using a Coulter Epics XL instrument. Compensation parameters for multiparametric studies were adjusted at the start of analysis, and correlated data of 10,000 events per sample were acquired with a live gate applied on lymphocytes, identified by forward- and side-scatter signals, and stored in list mode for subsequent evaluation. A logarithmic amplification was used for fluorescence intensity signals. The cursors were set on the isotype control stained cells, so that the positive region included less than 0.3% of the cells, and the same cursor settings were used to determine the antigen expression on positively stained cells in tubes containing the monoclonal antibodies.

Analysis was performed using Epics Elite software (Coulter). The latter records the mean fluorescence intensity (MFI) of the investigated antigens for each subset of cells resulting from the three-color analysis.

Statistical Analysis

The Student t test for independent variables was used to compare the percentages of the different T cell subsets in young and elderly donors as well as the MFI values of the investigated antigens on each lymphocyte subpopulation.

Results

The absolute number of total peripheral blood lymphocytes as well as the proportion of CD3+ T cells were substantially comparable in elderly and young donors (1,939.55 ± 628.36/mm^3 vs. 2,192.31 ± 462.71/mm^3 and 72.50 ± 12.01% vs. 71.60 ± 6.04%, respectively). However, several changes emerged in the representation of the different peripheral blood T lymphocyte subsets resulting from the three-color analysis (multiparametric flow cytometry analysis). Table 2 shows the percentages of peripheral blood T lymphocyte subsets from elderly individuals as compared with those from young donors.

The percentage of CD3+CD45RA+ naive T lymphocytes was significantly decreased, whereas the CD3+CD45RO+ memory subset was higher in old subjects as compared with the young ones. On the contrary, no significant differences in the representation of CD3+CD4+ helper/inducer and CD3+CD8+ suppressor/cytotoxic subpopulations between young and old individuals were detected. The percentage of CD3+ lymphocytes expressing the activation marker HLA-DR was increased in the elderly; this increased number of activated T lymphocytes concerned both CD4+ and CD4– (CD8) T cell compartments. The CD3+CD7– T lymphocytes were significantly increased in the elderly; moreover, both CD8+ and CD8– (CD4) subsets were expanded during senescence. Significantly lower percentages of T lymphocytes expressing the adhesion molecule CD62L were found in elderly donors as compared with the controls. On the contrary, no difference in the representation of CD3+ T cells expressing the adhesion molecule CD50 was detected.

Table 2. Peripheral blood T lymphocyte subset percentage in elderly and young people (mean ± SD)

Lymphocyte subsets	Percentage		p
	elderly	young	
CD3+CD4+ helper/inducer T cells	45.10±10.10	44.89±5.1	NS
CD3+CD8+ suppressor/cytotoxic T cells	24.1±10.10	20.9±4.8	NS
CD3+HLA-DR+ activated T cells	2.6±1.40	1.2±0.5	0.000
CD3+CD4+HLA-DR+ activated T cells	1.3±0.5	0.7±0.6	0.003
CD3+CD4- (CD8)HLA-DR+ activated T cells	1.5±0.6	0.5±0.4	0.000
CD3+CD7- T lymphocytes	14.6±5.85	8.35±2.1	0.000
CD3+CD7-CD8+ T lymphocytes	4.6±2.3	1.79±1.0	0.000
CD3+CD7-CD8- (CD4) T lymphocytes	10±5.1	6.1±1.1	0.007
CD3+CD45RA+CD45RO- naive T cells	15.3±9.2	24.7±5.3	0.001
CD3+CD45RA-CD45RO+ memory T cells	47.2±9.3	38.6±8.9	0.009
CD3+ T cells expressing CD50 adhesion molecule	70.19±13.6	69.48±4.55	NS
CD3+ T cells expressing CD62L adhesion molecule	30.84±12.23	48.79±6.03	0.000
CD3+CD2+CD56+ 'extrathymic' cytotoxic T cells	1.3±0.6	0.3±0.2	0.000
CD3-CD2+CD56+ NK T lymphocytes	6.9±3.7	4.1±2.8	0.020
CD3-CD7+CD8+ NK T lymphocytes	15.1±6.0	9.2±2.0	0.000

Table 3. MFI changes of T cell antigens in the elderly (mean ± SD)

Antigen	Cell subset	MFI		p
		elderly	young	
T cell antigen expressions which are significantly changed in the elderly				
CD3	CD3 T cells	17.20±2.26	19.85±2.35	0.002
CD3	CD45RO+ memory T cells	15.16±4.15	19.59±3.89	0.003
CD3	CD56+ 'extrathymic' cytotoxic T cells	17.86±5.93	12.79±6.77	0.022
HLA DR	CD3+ activated T cells	2.80±0.58	3.65±0.70	0.000
CD2	CD3+ T lymphocytes	3.80±0.75	2.59±0.43	0.000
CD7	CD3+ T lymphocytes	3.75±0.63	4.66±0.52	0.000
CD7	CD3-CD8+NK cells	7.90±2.75	11.74±3.53	0.000
CD8	CD3+CD7- T cell subset	4.12±1.93	2.61±1.27	0.010
CD50	CD3+ T lymphocytes	4.98±1.16	3.77±0.46	0.000
CD62L	CD3+ T lymphocytes	2.89±0.38	2.01±0.38	0.000
T cell antigen expressions which are unchanged in the elderly				
CD45RA	CD3+ naive T cells	2.80±1.09	2.40±0.34	NS
CD45RO	CD3+ memory T cells	8.11±1.85	9.10±1.78	NS
CD2	CD3-CD56+ NK cells	3.04±0.77	2.87±0.57	NS
CD56	CD3+ 'extrathymic' cytotoxic T cells	1.31±0.38	1.10±0.86	NS
CD56	CD3-CD2+ NK cells	1.63±0.33	1.96±0.96	NS
CD4	CD3+ helper/inducer T cells	3.69±0.34	3.53±0.35	NS
CD8	CD3+ suppressor/cytotoxic T cells	7.15±3.15	6.97±1.79	NS

Elderly donors showed an expansion of CD3-CD7+CD8+ natural killer (NK) cells. The percentage of NK cells appeared to be increased in the elderly also when selected on the basis of the expression of the NK marker CD56 (CD3-CD2+CD56+). Moreover, in the elderly also the CD3+CD2+CD56+ cytotoxic T lymphocytes were significantly increased over the conventional CD3+CD2+CD56- T cells.

Table 3 shows the density expression of lymphocyte antigens, expressed as MFI values, on peripheral blood T

lymphocyte subsets from elderly individuals as compared with that of young donors. The CD3 intensity expression on total T lymphocytes was lower in elderly as compared with young donors; moreover, in old donors, the expression of CD3 on CD45RO+ cells was decreased as compared with that of young controls. On the contrary, the CD3+CD2+CD56+ T cells from elderly donors exhibited a higher expression of CD3 as compared with young subjects. No differences in CD3 density on T lymphocytes expressing the adhesion molecules CD50 and CD62L were noted between young and old individuals. On the contrary, the expression of both CD50 and CD62L was higher on CD3+ T lymphocytes from elderly than on those from young subjects.

The intensity expression of CD45RA and CD45RO molecules on the naive and memory CD3+ T lymphocyte subsets was comparable in young and old donors, whereas the HLA-DR intensity expression on the cell membrane of activated T cells was decreased in elderly as compared with young controls. The MFI of CD7 antigen was significantly lower on both T (CD3+) and NK (CD3–) lymphocytes from old subjects as compared with young controls. No differences in CD4 and CD8 intensity expression were noted, except for a higher CD8 density on the CD3+CD7– T cell subset from elderly subjects. The CD2 molecule expression, expressed both on T (CD3+) and NK (CD3–) lymphocytes, was increased on T (CD3+) cells from elderly as compared with young donors, whereas no differences were noted on NK (CD3–) cells. No difference in CD56 antigen expression was shown on the NK cell membrane, as well as on CD3+ cytotoxic lymphocytes expressing this antigen, between old and young subjects.

Discussion

Several changes emerged from our study as far as the immune system of healthy elderly people is concerned, regarding cellular immunity and T cell phenotype: an increase in activated peripheral T cells [19], changes in the proportion of virgin/memory T cells [1], an increased number of NK cell activity markers [20], a decreased number of peripheral blood T cells expressing the adhesion molecule CD62L, and expansion of the CD3+CD7 T cell population, associated with a reshaping of the various receptor densities on the plasma membrane of different T lymphocyte subsets. All these immunological characteristics of T lymphocytes from elderly people could partially elucidate the mechanism underlying the age-related impairment of cellular immunity and would be in accord with the hypothesis that a complex remodeling of the T lymphocyte compartment occurs with age. The described changes in the composition of T cell subsets could be responsible for some functional characteristics of T cells from elderly people, such as the altered production of cytokines and the age-related impairment of T cell proliferation to new antigenic stimuli. In addition, the age-related modulation of receptorial molecules on the cell membrane could be another cause for T lymphocyte derangement, possibly through alteration of antigen recognition, adhesion mechanisms, cell-cell and cell-matrix interactions, and intracellular transduction signaling. Some of these antigen density changes have been noted before, e.g., increased expression of adhesion molecules [16–20]. The multiparameter flow cytometry methodology used in this study revealed changes in a substantially larger number of surface markers, some of which are restricted to fine subsets of T lymphocytes or activated lymphocyte subpopulations.

It is very likely that alterations in the function of the immune system due to defects in individual subsets are primary contributors to age-related immunodeficiency. To date, these studies have been done by performing cumbersome functional assays on a specific subset of cells. Unfortunately, determination of the functional capacity of all the subsets is prohibitive, both in terms of time and reagent requirements and in terms of available sample size required to perform these measurements. However, frequency and phenotype (i.e., antigen densities) of cell populations may provide alternative markers of the functional capacity of peripheral blood mononuclear cell subsets. The main age-related changes in T cell subsets could be interpreted in a unitary view of the peculiar attitude of the immune system in the elderly. Both T cell subset distributions and immunophenotypical changes expressed as alterations in antigen densities on T cell membrane are the consequence and/or the result of compensation mechanisms for the deficient regeneration and aging of the immune system. The T cellular compartment of elderly individuals reflects in fact the physiological thymic involution typical of senescence and the accumulation of continuous immunological experiences and contacts with an ever-growing number of antigens. The generation of new lymphocytes is compromised by the deficiency of a thymic tissue at the maximum grade of its functionality, and an accumulation of memory-primed T lymphocytes is documented. Also the CD7 downregulation and the increase of CD7– T lymphocytes reflect the greater number of mature and activated T lymphocytes. It is well known that the CD7 molecule, overexpressed on the cell mem-

brane of immature T lymphocytes, is gradually downregulated during maturation and activation till the final stage of memory T cells. CD3+CD7– T lymphocytes are in fact mainly activated and memory cells at a fully differentiated maturational stage [11]. Moreover, the subsets CD3+CD7– are alterated not only in the number but also in the expression of CD8 that is upregulated in the elderly. Similar considerations can explain the decreased expression of the receptorial molecule CD3 of the T lymphocytes of elderly subjects as a consequence of the generalized T cell activation and the decrease of naive cells characteristic of the elderly. CD3 antigen is in fact part of the T cell receptor complex; it is downregulated after activation and is lower in memory than in naive T cells. A decreased T cell receptor expression after activation and an impaired proliferation of T cells from old mice after stimulation with concanavalin were demonstrated which could be ascribed partly to altered expression of various surface molecules, receiving antigenic or various intercellular positive and negative signals. The decreased expression of CD3 on memory cells from old donors suggests an altered activation process of these lymphocyte compartments in the elderly, i.e., a sort of hyperactivated state of each lymphocyte which underwent exhaustive stimulation. This could contribute to the altered function and proliferative response of memory T cells typical of senescence [1, 5, 21, 22]. The decreased HLA-DR intensity expression of T cells in the elderly seems to confirm an anomaly of the activation processes of the T cell compartment. The increase of T lymphocytes that express NK markers is interpreted as a compensation for the deficit of cytotoxic cellular immunity and NK activity on the one hand and for the decreased thymic capacity to generate new conventional T lymphocytes on the other [8]. In fact, both NK T lymphocytes and CD3+CD56+ have probably an extrathymic origin, with specific and separate maturation pathways. A decreased CD7 expression intensity is typical of the NK cells in elderly persons and perhaps is a marker of activation of this cellular compartment. However also this receptorial modulation could contribute to the deficit of NK function documented by some authors [11, 23]. The increased expression of the CD3 on the T CD56+ lymphocytes could correspond to a further mechanism of compensation, in addition to the numerical increase of these subsets, for the decreased conventional T lymphocyte function. In any case, these data confirm that besides the simple numerical alteration, the specific T cellular subsets express also the intrinsic phenotypical characteristics in elderly persons in comparison to the young. For example the CD2 is increased on T lymphocytes and not on the NK cells of the elderly, confirming that the antigenic modulations we have documented are typical of single cellular populations. The CD3 antigen exerts an important role in adhesion and activation processes. Its expression on the cell surface progressively increases with maturation and activation [24, 25]. CD2 upregulation may be important during senescence: by interaction with intercellular adhesion molecules, CD2 can increase the adhesion capability and, therefore, the immunological function of T cells in healthy elderly subjects.

In any case, the greater intensity expression on T cells fits with the increased expression of the other adhesion molecules, CD50 and CD62L, detected in T lymphocytes from elderly subjects and could, therefore, be interpreted as a mechanism of compensation to increase the adhesion and interaction cellular functions. Cell adhesion molecules (CAM) are membrane-bound proteins that play a crucial role in mediating cell-cell interactions [26, 27]. Aging of immune cells seems to be associated with increased epitope density expression of several CAM which could very likely result in a greater capacity to adhere [16]. An increased expression of CD11a has been described in lymphocytes from a few donors older than 60 years [28]. LFA-1^{high} cells represent the large majority of CD8+ T lymphocytes, including those that are CD45RA+ [28, 29]. The same phenomenon has been observed in cells from healthy centenarians who, moreover, have among the same population of virgin CD8+ cells increased levels of another CAM, i.e., CD49d [16]. The adhesion molecules CD50 and CD62L, especially expressed on the resting T lymphocytes, are downmodulated on activated and memory T lymphocytes [30, 31]. Our finding of a decreased percentage of T lymphocytes, expressing the CD62L adhesion molecule, agrees with these observations. The higher expression density of the adhesion molecules on T lymphocytes in the elderly could represent a mechanism of compensation for the decreased adhesion capacity of T lymphocytes in old people. CD62L is specifically downregulated immediately after stimulation of T cells through a specific proteolytic event [32]. It is possible that the increased expression reflects a low level of lymphocyte activation or alternatively some alterations in enzymatic events following stimulation. The change in density may reflect a difference in the regulation of expression of such an antigen on a per-cell basis. This change may well reflect (even if not cause) functional differences. For instance, upregulation of CD62L could alter homing of T and B cells bearing this integrin, inducing an abnormally increased compartmentalization of lymphoid cells. Since CD62L is essential for lymphocyte

homing, altered expression of this molecule could lead to altered lymphocyte recirculation. As a partial explanation, the expression of several CAM and that of other molecules related to the immunoglobulin superfamily may be modulated by a variety of soluble mediators, including proinflammatory cytokines [33]. During human aging and longevity, a complex modification of the cytokine network occurs which could account, at least in part, for the modulation of CAM expression. According to Cossarizza et al. [16], one could interpret the modulation of CAM as an effort of the immune cells to cope with a decreased responsiveness to given stimuli or with a greater requirement for activation signals. Also the expression of the adhesion molecule CD50 was higher on T lymphocytes in the elderly. Immunosenescence and longevity, as suggested also from our study on healthy and well-selected elderly subjects, including centenarians, represent a complex process based on a new and peculiar reequilibrium of the immune system. We think that it is essential to understand these complex events with the aim of better diagnosing and in particular better treating and preventing the true pathological conditions in the elderly so as to ensure a physiological immunosenescence and a healthy longevity.

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