

Lymphocyte Subsets and Natural Killer Cell Activity in Healthy Old People and Centenarians

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The contribution of the immune system to healthy aging and longevity is still an open question. For this reason, several immune parameters (T, B, and natural killer [NK] cell subsets; non-major histocompatibility complex [MHC]-restricted cytotoxic activities, ie, natural and redirected killing [RDK] activities) were studied in a total of 138 healthy subjects of different ages, from 4 to 106 years of age, including 26 centenarians. The major age-related modifications were the following: (1) a decrease in the absolute number of T lymphocytes (CD3⁺), involving both CD4⁺ and CD8⁺ subsets, accompanied by a marked concomitant increase in the number of activated T cells (CD3⁺, HLA-DR⁺); (2) a marked decrease in the number of B lymphocytes (CD19⁺); and (3) an increase in the number of cells with markers of NK activity and of T lymphocytes able to mediate non-MHC-restricted cytotoxicity. These modifications linearly progressed with age and centenarians fol-

lowed the trend, suggesting that their immune system did not escape the aging process. However, other immunohematologic parameters (number of red blood cells, platelets, and leukocytes) and important immune functions, such as cytotoxic activities (NK and RDK cell activities), were well preserved throughout life until the last decades of life. Unexpectedly, in apparently healthy middle-aged subjects, a decrease of cytotoxic activities was observed in comparison with those of both young controls and centenarians. In conclusions, our data suggest that in centenarians some immune responses are kept at a high level of efficiency, likely contributing to their successful aging. However, this selected group of people does not escape the aging process, as shown by the progressive derangement of a variety of immune parameters.

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ACCORDING TO SOME investigators, there is a derangement of some immune responses with age that can contribute to and can be a predictor of the increased age-related risk of morbidity and mortality.¹⁻³ However, the careful selection of healthy aged subjects suggests that many immune parameters are well conserved throughout life.⁴⁻⁶ Indeed, most immunogerontologic studies focus on subjects aged 70 to 80 years, in comparison with younger donors, usually 20 to 40 years old. In this way, a significant part of human life, ie, the first 20 and the last 20 to 30 years, is neglected. In particular, data regarding subjects more than 90 to 100 years of age are scanty,⁷ despite their increasing number.⁸⁻¹⁰ We addressed this problem by studying peripheral blood lymphocyte subpopulations and cytotoxic activities in a total of 138 subjects aged 4 to 105 years and by paying particular attention to their health status. The results obtained, likely representative of physiologic aging, show that a profound reshaping of T, B, and natural killer (NK) cell subsets occurs throughout life. Important immune parameters are well preserved in centenarians whose immune system, however, does not escape the aging process.

MATERIALS AND METHODS

Subjects. The study was performed with the informed consent of the donors or of their parents. A total of 138 subjects was studied; 112 were less than 100 years old, and 26 were more than 100 years old. All of the 112 control subjects, whose age ranged from 4 to 98 years, were in healthy condition, according to accurate clinical investigations and to the usual hematochemical parameters. In this group, all the people older than 65 years fulfilled the strict inclusion criteria of the SENIEUR Protocol,^{4,6} and were chosen among a population of more than 300 old citizens.

Centenarians had a mean age of 100.7 ± 0.3 years (range, 100 to 106 years; 3 men and 23 women). All of them were in good clinical condition, without any relevant acute or chronic disease that could affect the immune system, and mentally competent to give informed consent. In particular, none of the subjects had cancer; was suffering from serious cardiac, brain, or kidney disease; or was tak-

ing drugs known to affect the immune system. In addition to a complete social and medical history, a physical examination was performed. Moreover, several hematochemical parameters (blood concentrations of glucose, cholesterol, sodium, potassium, triglyceride, bilirubin, albumin, Igs, uric acid, alkaline phosphatase, glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, lactic dehydrogenase, creatinine phosphokinase, iron, etc) were determined.

Owing to a shortage of cells, cytofluorimetric analysis was not performed in all subjects, but only in 11 centenarians and in most of the other donors.

Cytotoxicity studies were performed in all of the centenarians, in 25 healthy middle-aged subjects with a mean age of 63.5 ± 1.4 years (range, 50 to 68 years), and in 25 healthy young donors with a mean age of 28.4 ± 0.5 years (range, 19 to 36 years).

Hematologic investigations. The total number of leukocytes, red blood cells, hemoglobin (Hb), hematocrit, and platelets was determined in all subjects by means of a Coulter counter (Coulter, Hialeah, FL). The leukocyte differential count was performed by optic microscopy after staining of a smear of peripheral blood with May-Grünwald-Giemsa, following standard methods.

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Submitted April 21, 1993; accepted June 30, 1993.

Supported by grants from Consiglio Nazionale delle Ricerche (P.F. "Invecchiamento") paper No. 93-1-328 to P.S., U.F., and C.F., and from M.U.R.S.T. (40% and 60%) to P.S., M.P., and C.F.

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0006-4971/93/8209-0015\$3.00/0

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Immunologic assessment. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque density centrifugation from freshly drawn venous blood collected around 8 AM from a centenarian, a middle-aged subject, and a young control, all on the same day. After washing with phosphate-buffered saline (PBS), the cells were resuspended in RPMI 1640 with 10% heat-inactivated human AB serum, 2 mmol/L L-glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin, hereafter referred to as complete medium.

Phenotype analysis and flow cytometry. The phenotypical analysis of peripheral blood lymphocytes (PBL) was performed on whole blood samples as previously described.¹¹

The following monoclonal antibodies (MoAbs), purchased from Becton Dickinson (San Jose, CA), directly conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), were used to analyze the surface antigens of PBL: anti-CD3 (anti-Leu4), recognizing all T cells; anti-CD4 (anti-Leu3), reactive with helper/inducer T-cell subset; anti-CD8 (anti-Leu2a), recognizing cytotoxic T-cell subsets and some NK cells; anti-HLA-DR, recognizing B cells and activated T lymphocytes; anti-CD16 (anti-Leu11a or anti-Leu11c), recognizing the low-affinity receptor for FcIgG, reactive with a subset of cells with NK activity and neutrophils and with lymphocytes also expressing CD57 and/or low-density CD8 antigens; anti-CD56 (anti-Leu19), recognizing the neural cell adhesion molecule (N-CAM) molecule, reactive with resting and activated CD16⁺ cells and with a small percentage of CD3⁺ lymphocytes, which are considered to be a subset of cytotoxic T lymphocytes that mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity; anti-CD57 (anti-Leu7), reactive with a subset of cells with NK activity, with a subset of T lymphocytes, and with some CD8⁺ cells; anti-CD19 (anti-Leu12), reactive with B lymphocytes; anti-CD14 (anti-LeuM3), reactive with monocyte-macrophages and used to evaluate the percentage of monocytes present in the forward and orthogonal light scatters that were used to identify the lymphocyte gate during the cytofluorimetric analysis. IgG1, IgG2a, IgG2b, and IgM conjugated with FITC or with PE (Becton Dickinson or Coulter) were used as negative controls for nonspecific binding.

Cytofluorimetric analysis was performed with a FACSCAN cytofluorimeter (Becton Dickinson), as described elsewhere.¹² A minimum of 10,000 cells per sample was analyzed.

Cytotoxicity assays. NK activity was measured in a 4-hour ⁵¹Cr release cytotoxicity assay using the K562 human erythroleukemia cell line as a target.¹² Five thousand ⁵¹Cr-labeled K562 cells were added to each well containing various concentrations of PBMC. After a 4-hour incubation, the release of ⁵¹Cr into the supernatants was determined in triplicate at effector/target ratios of 6/1, 12/1, 25/1, and 50/1. Maximum and spontaneous ⁵¹Cr release values were obtained by adding 0.2 mL of saponin (Emolis; Flow, Irvine, CA) or complete medium, respectively, to microtitre wells, containing 5 × 10³ labeled target cells. The mean corrected percentage lysis was calculated by the following formula: Percentage of lysis = (cpm experimental - cpm spontaneous release)/(cpm maximum release - cpm spontaneous release) × 100.

Data on NK cytotoxicity are expressed in terms of lytic units₄₀ (LU₄₀) per 10⁷ cells, calculated according to Pross et al,¹³ on the basis of the dose-response curve. One LU₄₀ corresponds to the number of effector cells necessary to lyse 40% of the targets.

Redirected killing (RDK) was performed by using the mouse mastocytoma cell line P815 as a target.¹⁴ Briefly, ⁵¹Cr-labeled P815 cells were coated with saturating concentrations of anti-CD16 MoAb (anti-Leu11c; kindly provided by Dr N. Damle, Cetus Co, Emeryville, CA), washed, and mixed with different concentrations of PBMC. After a 4-hour incubation, ⁵¹Cr release into the superna-

tants was determined in triplicate at effector/target ratios of 6/1 to 50/1. Maximum and spontaneous ⁵¹Cr release values and mean percentage lysis were obtained as described for the NK activity test. Data are expressed as LU₄₀, as described above.

Statistical analysis. Statistical analysis was performed by linear regression analysis and two-tailed Student's *t*-test.

RESULTS

The major age-related immunologic variations observed in our healthy population were the following (Figs 1 through 3): (1) a decrease in the absolute number of lymphocytes and T lymphocytes (CD3⁺), involving both CD4⁺ and CD8⁺ subsets, accompanied by a marked concomitant increase in the number of activated T cells (CD3⁺, HLA-DR⁺); (2) a marked decrease in the number of B lymphocytes (CD19⁺); (3) an increase in the number of cells with markers of NK activity (CD57⁺, both percentage and absolute number; CD16⁺, percentage; CD56⁺, percentage; CD16⁺,CD57⁻, both percentage and absolute number; CD16⁺,CD57⁺, neither percentage nor absolute number; CD16⁻,CD57⁺, percentage); and (4) an increase in the percentage and absolute number of CD3⁺,CD56⁺ cells, namely of T lymphocytes able to mediate non-MHC-restricted cytotoxicity.

It is interesting to note that in several cases the variations of the absolute number of the different subsets did not mirror their percentage (CD3⁺, CD4⁺, and CD8⁺ cells) and vice versa (CD16⁺, CD56⁺, and CD16⁻,CD57⁺ cells).

To ascertain whether the increased number of cells with NK markers with age was accompanied by a concomitant functional efficiency, an NK cell activity test was performed.

Table 1 shows that NK activity is well preserved throughout the entire life span, namely from young subjects to centenarians. Unexpectedly, NK activity of middle-aged controls was significantly lower in comparison to that in both young subjects and centenarians. It is interesting to note that a well-preserved NK activity is also observed when cells from old siblings of centenarians are used (data not reported). The same pattern was observed when another type of cytotoxicity, ie, RDK, was assessed. In this case as well the activity of cells from centenarians was still well preserved.

Besides NK and RDK cell activities, other immunohematologic parameters were well preserved in centenarians. In particular, the hematopoietic function of centenarians was very well preserved. Table 2 shows that Hb concentration, platelet number, total white blood cell (WBC) count, and percentages of neutrophils, monocytes, eosinophils, and basophils were not significantly different from the young or middle-aged groups. Indeed, only lymphopoiesis was severely affected in centenarians, as shown by the highly reduced number of lymphocytes.

DISCUSSION

In the literature, the deterioration of the immune system with age is controversial. Early studies suggested that major age-related changes, particularly of the T-cell compartment,

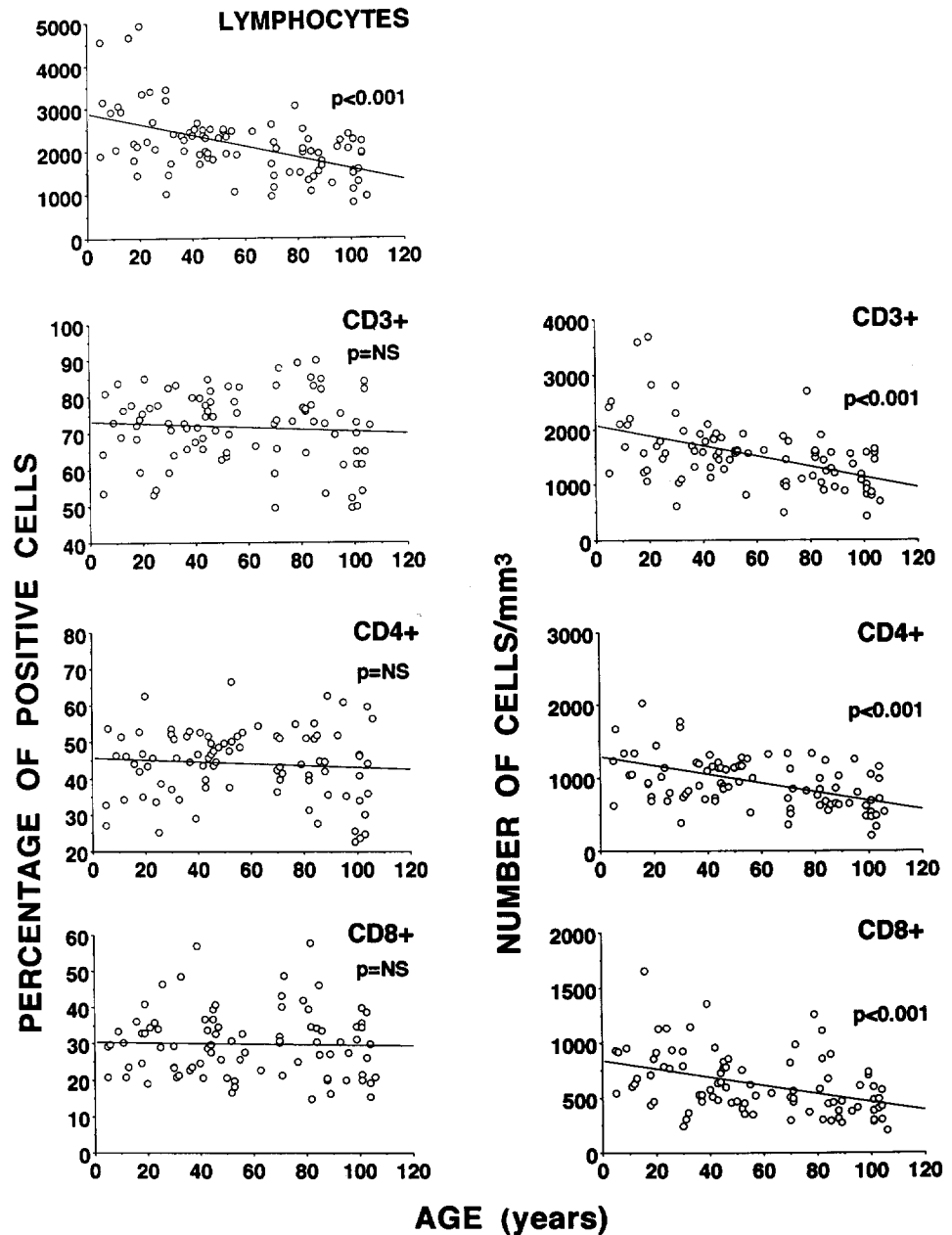


Fig 1. Phenotypic analysis of PBL from the subjects studied. Total number of lymphocytes per microliter and absolute numbers and percentages of CD3⁺, CD4⁺, and CD8⁺ cells are shown. The regression line is indicated within each panel and *P* values were calculated by the linear regression analysis.

were present in people older than 65 years of age.¹⁵ Recently, some investigators argued that in these studies there was a major bias mainly due to the poor attention paid to admission criteria in gerontologic studies. Indeed, chronological age is not the only or major variable to be considered; the health status of the subjects must be evaluated with strict criteria. The SENIEUR Protocol was proposed to avoid this problem and to offer the scientific community a way to compare gerontologic studies, particularly those focussed on the immune system.

Studies in which these criteria were fulfilled showed that the deterioration of the immune system is less pronounced than previously thought, even if several parameters do change with age.⁴⁻⁶ However, even in studies fulfilling the

aforementioned admission criteria, scanty data were offered concerning people older than 85 to 90 years of age. Moreover, it is generally admitted that the human life span is of the order of 115 to 120 years.⁸⁻¹⁰ Thus, a significant part of human life, the last 25 to 30 years, is ignored or not properly studied.

The data presented in this report show that a modification of all major lymphocyte subsets occurs with age and that this is particularly evident in centenarians.

A progressive and consistent age-related decrease of all major T subsets, ie, CD4⁺ and CD8⁺, emerges. This is in apparent contrast with previous reports suggesting that the decline of T cells is ascribable to a particular subset.¹⁶ Indeed, a comparison with previously published reports is dif-

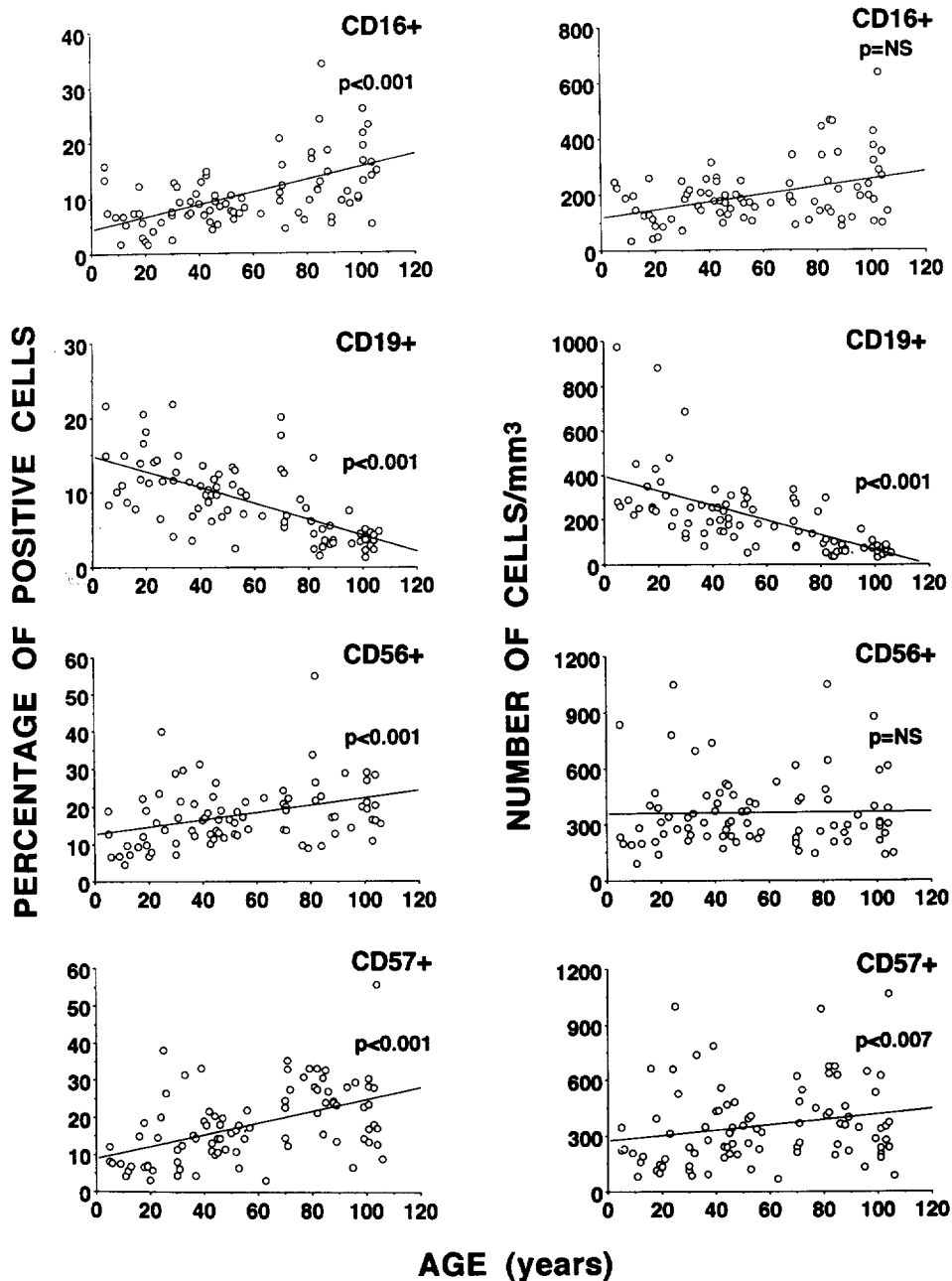


Fig 2. Phenotypic analysis of PBL from the subjects studied. Absolute numbers and percentages of cells with markers related to NK activity (CD16, CD56, and CD57) and B lymphocytes (CD19⁺ cells) are shown. The regression line is indicated within each panel and *P* values were calculated by the linear regression analysis.

difficult, owing to the fact that in some cases only the percentages of T, B, and NK cells are reported.^{17,18}

An activation of the immune system of unknown nature occurs with age, as suggested by an increased number of activated T cells (CD3⁺, HLA-DR⁺ lymphocytes) as reported in Down's syndrome, in which an acceleration of the aging process has been observed.^{11,12,19} However, no correlation appears to exist between activated T cells, which progressively increase with age, and memory (CD45RO⁺) T cells, which are substantially stable from 30 years of age onwards.²⁰

A characteristic of the aging process is the dramatic decrease of circulating B lymphocytes, accompanied by an

increased serum level of some Ig classes and IgG subclasses²¹ and by the almost complete absence of organ-specific autoantibodies.²² Studies on immune districts other than peripheral blood are needed to clarify this apparent paradox. The age-related increase of NK cells is well documented.^{23,24} A detailed cytofluorimetric analysis allowed us to demonstrate an age-related increase of cells with high NK activity (CD16⁺, CD57⁻).²⁴ On the contrary, cells with intermediate (CD16⁺, CD57⁺) or low (CD57⁺, CD16⁻) NK activity²⁵ showed only minor modifications. In centenarians, the increase of the high-activity NK subset is mirrored by very well-preserved cytotoxicity, measured by both NK and RDK assays. The RDK test was performed to evaluate the

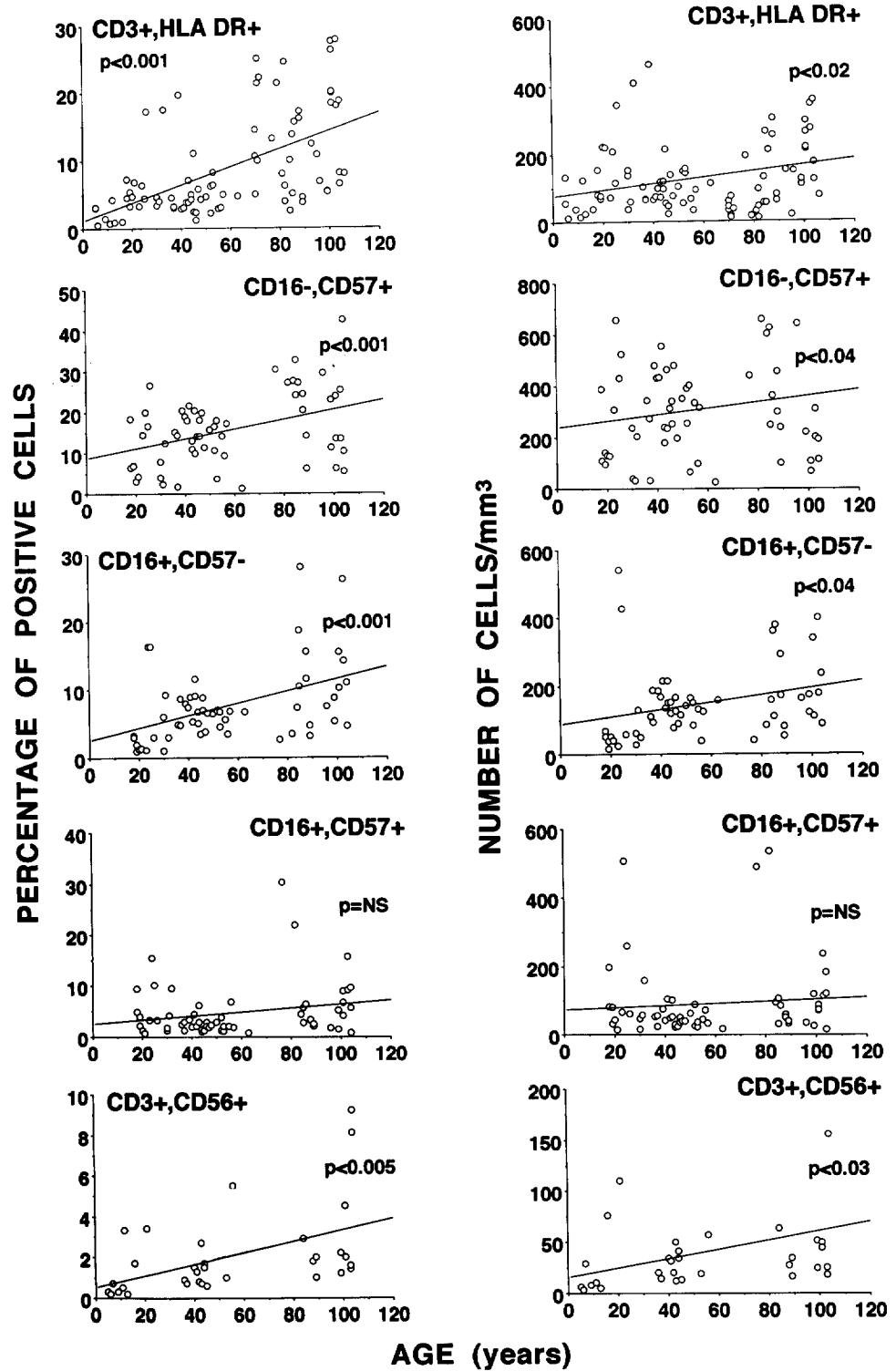


Fig 3. Phenotypic analysis of PBL from the subjects studied. Absolute numbers and percentages of activated T lymphocytes (CD3⁺,HLA-DR⁺), of NK cells (recognized by anti-CD16 and/or anti-CD57 MoAbs), and of non-MHC-restricted cytotoxic T lymphocytes (CD3⁺, CD56⁺) are shown. The regression line is indicated within each panel and P values were calculated by the linear regression analysis.

intrinsic cytotoxic capacity of killer cells, independent of their ability to properly recognize adequate structures on the membrane of target cells. Conflicting data are present in the literature regarding NK activity and aging.^{24,26} It is likely that the age-related decreased NK activity reported by some investigators can be ascribed to selection bias, as suggested

by the fact that people selected according to strict criteria (SENIEUR Protocol) do not present such a diminution (Lighthart et al⁵ and unpublished data). However, other factors can also play a role. In fact, an unexpected finding was the significantly decreased NK activity in the group of apparently healthy middle-aged subjects. It has been noted that

this period of life is poorly studied, and an effort should be made to cover this gap.²⁷ This age coincides with great changes of the neuroendocrine system. Indeed, the number of observations on unexpected findings in middle-aged people is increasing. For example, it has been recently reported that both luteinizing hormone burst frequency and total secretion are lower in middle-aged subjects than in younger people.^{28,29} It is known that NK activity is very sensitive to neuroendocrine changes,³⁰ and further studies are needed to verify the hypothesis that such a decrease of NK activity in middle-aged subjects is related to this phenomenon. Recent data suggest that a persistently low NK activity is a predictor of morbidity.³¹ Conversely, it can be speculated that well-preserved NK activity can help in reaching far advanced age in good conditions. The age-related increase of cells bearing NK markers and of non-MHC-restricted T lymphocytes could be interpreted as a compensatory mechanism to cope with the decrease of T cells probably related to the thymic involution.

The results on the immune parameters of healthy centenarians deserve particular attention. A consistent percentage (about one-third) of centenarians are in relatively good clinical condition, despite their age. Thus, the study of such very old healthy people is particularly interesting from the biomedical point of view because it could help in identifying the crucial mechanisms responsible for the maintenance of body homeostasis. The clinical history of these people is in most cases impressively free of major age-related diseases, suggesting that the most important mechanisms responsible for body homeostasis at a cellular and supracellular level (immune system) should continue to work at a remarkably high level of efficiency to assure survival in good general conditions.³² Indeed, the data reported here suggest that this is the case, and that important hematologic and immune parameters are very well preserved in people over 100 years of age, even if their immune system does not escape the aging process.

In conclusion, our data suggest that the three major lymphoid cells present in the peripheral blood are all affected by age. T and B cells and their subsets decrease, whereas most cells with NK markers and particularly those with high NK activity increase with age. These modifications linearly progress with age, and centenarians follow the trend and help in assessing its direction. The high level of functional perfor-

Table 2. Hematologic Parameters of Centenarians, Middle-Aged Subjects, and Young Donors in Whom Cytotoxicity Tests Were Performed

	Centenarians	Middle-Aged	Young
No. of subjects	26	25	25
Age (yr)	100.9 ± 0.3	57.9 ± 1.2	27.2 ± 0.8
WBC/ μ L	5,540 ± 269	5,000 ± 318	5,700 ± 280
Lymphocytes (%)	29.6 ± 1.8*	36.0 ± 1.8	40.0 ± 2.6
Neutrophils (%)	62.3 ± 2.0	57.1 ± 2.1	53.0 ± 2.8
Monocytes (%)	4.5 ± 0.4	4.0 ± 0.2	3.8 ± 0.4
Eosinophils (%)	2.4 ± 0.4	2.4 ± 0.3	2.4 ± 0.5
Basophils (%)	1.0 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
Hb (g/dL)	12.7 ± 0.2	12.8 ± 0.3	13.2 ± 0.3
Platelets ($\times 10^3/\mu$ L)	238 ± 19	214 ± 14	262 ± 10

Values are the arithmetic mean \pm SEM.

* $P < .002$ v young donors and $P < .02$ v middle-aged subjects by Student's t -test.

mance characteristic of NK cells from centenarians is likely to be the result of a peculiar combination of genes. Indeed, the analysis of HLA antigens suggests that some haplotypes are significantly more represented in the centenarians we have studied.³³ Further studies are needed to clarify this point.

ACKNOWLEDGMENT

The authors gratefully thank Dr Elizabeth A. Cristofalo for critically reading the manuscript.

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Table 1. Cytotoxic Capability of PBMC From Centenarians, Middle-Aged and Young Donors

Test	C	MA	Y
NK	54.7 ± 9.6	33.4 ± 3.9	63.3 ± 8.9
RDK	75.0 ± 18.5	66.3 ± 9.5	74.9 ± 18.1

Data are expressed as LU₄₀ per 10⁷ cells (mean \pm SEM) according to the formula quoted in Materials and Methods. Tests were performed on 25 Y, 25 MA, and 26 C (NK cell activity [NK]), or on 10 Y, 9 MA, and 9C (RDK). Statistical analysis was performed by two-tailed Student's t -test: C v MA: NK, $P < .05$; RDK, $P = NS$; C v Y: NK, $P = NS$; RDK, $P = NS$; MA v Y: NK, $P < .003$; RDK, $P = NS$.

Abbreviations: C, centenarians; MA, middle-aged donors; Y, young donors; NS, not significant.

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