Immunology 1986 **59** 353–357

The expanded null cell compartment in ageing: increase in the number of natural killer cells and changes in T-cell and NK-cell subsets in human blood

G. J. LIGTHART, P. C. VAN VLOKHOVEN, H. R. E. SCHUIT & W. HIJMANS *Department of Internal Medicine, Study Group for Medical Gerontology, University Hospital, Leiden, The Netherlands*

Accepted for publication 4 July 1986

SUMMARY

Analysis of the subpopulations of mononuclear cells in human blood in ageing has revealed a striking increase in the number of null cells, defined as non-T, non-B, non-monocyte cells, and a decrease in the number of T and B cells. By using recently developed monoclonal antibodies against natural killer cells in combination with T-cell markers in two-wavelength immunofluorescence, we were able to define 13 subpopulations of mononuclear cells and compare them in two groups of persons, respectively aged 25–34 and 75–84 years, all fulfilling the stringent admission criteria for immunogerontological studies described in the SENIEUR protocol, and thus all to be considered as optimally healthy and immunologically uncompromised. We found that the increased null cell population in the aged is a result of an increase in the numbers of NK cells, mostly the $CD16^+Leu7^+$ subset. The number of $CD8^+$ suppressor/cytotoxic cells is decreased. This is due to a decrease of the number of $CD8^+Leu7^-$ cells. All NK and T-cell subsets bearing the Leu7 antigen, namely $CD16^+$ $Leu7^+$, $CD4^+Leu7^+$ and $CD8^+Leu7^+$, are increased. These changes can be due to defects of the ageing immune system, but they can also represent the optimal state of the immune system in the healthy aged and may be linked to survival. These values can be used as reference values for the 75–84 years age group and serve to monitor attempts to reconstitute the immune defects in ageing.

INTRODUCTION

The immune system in ageing man is known to show age-related defects (reviewed by Kay & Makinodan, 1981), mainly in the T-cell system. The study of the subpopulations of mononuclear cells in human blood has yielded conflicting results. This mostly seems to be due to the heterogeneity of the subjects admitted to immunogerontological studies. We recently investigated a group of aged persons fulfilling the admission criteria described in the SENIEUR protocol (Ligthart *et al.*, 1984) and because of the strictness of these criteria these individuals should be considered as optimally healthy and immunologically uncompromised. In this group we found a stiking increase in the number of null cells, defined as non-T, non-B, non-monocyte (Ligthart, Schuit & Hijmans, 1985). We also found a slight decrease in the number of T cells, mostly of the T suppressor/cytotoxic subpopulation, and a slight decrease in the number of B cells. The number of monocytes remained constant.

Null cells, also called third population cells (Frøland & Natvig, 1973), L cells because of their labile Fc receptor (Horwitz & Lobo, 1975) or UMC (undefined mononuclear cells, Schuit & Hijmans, 1980), are known to be a heterogenous population of cells and to exert most of natural killer (NK) function (Jondal & Pross, 1975). Recently, several monoclonal antibodies have been specifically raised against NK cells. B73.1 (CD16, Leu11c) recognizes the NK cell-specific Fc receptor (Perussia *et al.*, 1983) and does not react with mature T cells, B cells, monocytes or granulocytes (Trinchieri & Perussia, 1984). Leu7, also known as HNK-1, reacts with a morphologically defined subset of large granular lymphocytes, part of which are NK cells but part are T cells lacking NK function (Abo & Balch, 1982).

In this study we analysed subpopulations of mononuclear cells by using B73.1 (Leu11c), Leu7 and markers for T-cell subsets in single and in double staining with two-wavelength immunofluorescence in combinatioin with phase contrast microscopy. This technique enables us to distinguish 13 different subsets of mononuclear cells. We found that virtually all the cells in the null cell compartment as defined above react with B73.1 and can thus be identified as NK cells. The decrease in the number of T cells is caused by a decrease of the $CD8^+$ suppressor/cytotoxic subset, but this decrease is confined to the $CD8^+Leu7^-$ cells. All NK and T cells subsets bearing the Leu7 antigen, namely $CD16^+Leu7^+$, $CD4^+Leu7^+$ and $CD8^+Leu7^+$, show a significant increase.

Correspondence: Dr Gerard J. Ligthart, Department of Internal Medicine, University Medical Center, Building 1, Cl-38, Rijnsburgerweg 10, 2300 AA Leiden, The Netherlands.

MATERIALS AND METHODS

Persons studied

Thirty-three volunteers (14 males and 19 females) aged 75–84 years (mean, 80 years) and 35 volunteers (19 males and 16 females) aged 25–34 years (mean, 28 years), all of Dutch caucasoid origin, were admitted to the study. The older subjects were all living in independent units of homes for the aged; the young controls were recruited among hospital and laboratory staff. All fulfilled the strict admission criteria described in the SENIEUR protocol (Ligthart *et al.*, 1984). This protocol details exclusion criteria based on clinical information, laboratory data with age-dependent reference ranges, and it sets rules for the limitation of pharmacological interference.

Preparation of mononuclear cells

The preparation and the handling of the cells for immunofluorescence have been described in detail in previous reports (Schuit, Hijmans & Asma, 1980; Ligthart, Schuit & Hijmans, 1985). The first antibody layer was applied as mentioned before, this time using combinations of two antisera with different Ig-class or subclass simultaneously. For the second antibody combination we used a mixture of two goat anti-mouse Ig class and subclass specific conjugates, each labelled with a different fluorochrome.

Microscopy and quantification of subpopulations

The cells were first examined for morphology in a Leitz Dialux® microscope (Wetzlar, FRG) using low-voltage transmitted light and an oil-immersion phase contrast objective lens ×63/1·30 with oculars ×6·3. The transmitted beam was then replaced by incident narrow-band excitation light using a Ploem opak 2·4 illuminator containing the filter sets suitable for selective visualization of FITC and TRITC fluorescence. A mercury HBO 100 W lamp served as the light source. At least 200 mononuclear cells were classified according to morphology and fluorescence. In the double marker studies, at least 100 cells positive for one fluorochrome were examined for reactivity with the other label using the two-wavelength method. Each subset was expressed as a percentage of total mononuclear cells. To obtain the absolute number of cells per subset, a leucocyte count was performed on whole blood by Coulter counter® (Coulter Electronics, Hialeah, FL) and a leucocyte differentiation count was performed with a Haemalog D (Technicon Instruments, Tarrytown, NY). The absolute number of mononuclear cells was calculated from these data, and multiplied by the percentage of cells in a subset to obtain absolute numbers of cells per subset.

Antisera

The antisera and fluorescent conjugates used, their Ig-classes, subclasses, and working dilutions are given in Table 1. The goat anti-mouse Ig (GAM/Ig) conjugates used as second antibodies were tested for specificity using the indirect technique on formaldehyde-fixed human mononuclear cells after incubation with mouse monoclonal antibodies of different Ig-classes and subclasses. No cross-reactivity between the subclass-specific anti-Ig was observed. The absence of cross-reaction with human Ig was established on the same substrate in a single-staining procedure.

Detection of subpopulations

Null cells, defined as non-T, non-B, non-monocyte cells, were counted as cells not reacting with Leu4-FITC, nor with HLA-DR-TRITC in the two-wavelength method. DR-negative monocytes were excluded by morphology in phase-contrast microscopy. Natural killer (NK) cells were defined by their reactivity with the monoclonal antibody B73·1 (CD16$^+$) (Perussia *et al.*, 1983) kindly provided by Dr G. Trinchieri. The mononuclear cells were also analysed with the monoclonal antibody Leu7, also known as HNK-1, reported to react with large granular lymphocytes and also reacting with subsets of cells in both T and NK compartments (Abo & Balch, 1982). T cells (CD3$^+$) were enumerated with Leu4, T helper/inducer (CD4$^+$) with Leu3a. T suppressor/cytotoxic cells (CD8$^+$) were

Table 1. Antisera

Antibody specificity and cluster designation	Ig class/ subclass	Working dilution*	Fluorochrome	Assigned specificity and source
B73.1 (CD16)	Mouse IgG1	40	—	NK cells†
Leu7	Mouse IgM	200	—	Part of NK and T cells‡
Leu4 (CD3)	Mouse IgG1	20	FITC	T ly‡
Leu3a (CD4)	Mouse IgG1	80	—	T helper/inducer‡
Leu2a (CD8)	Mouse IgG1	80	—	T suppressor/cytotoxic‡
FK18 (CD8)	Mouse IgG3	400	—	T suppressor/cytotoxic§
HLA-DR	Mouse IgG2a	100	TRITC	Bly,act.Tly, monocytes‡
GAM/IgG1	Goat IgG	64	FITC	Mouse IgG1¶
GAM/IgG3	Goat IgG	24	TRITC	Mouse IgG3¶
GAM/IGM	Goat IgG	24	TRITC	Mouse IgM¶

* Reciprocal value.
† Dr G. Trinchieri, Wistar Institute, Philadelphia, PA.
‡ Becton Dickinson, Sunnyvale, CA.
§ Dr F. Koning, Leiden University Medical Centre, Leiden, The Netherlands.
¶ Nordic Immunological Laboratories, Tilburg, The Netherlands.

identified with the monoclonal antibodies Leu2a or FK18. The latter antibody precipitates the same molecule of approximately 33, 000 MW as Leu2a (Koning, 1982), but our tests show that it recognizes another epitope of this molecule: when mononuclear cells are incubated with a mixture of Leu2a and FK18, followed by a mixture of the appropriate GAM/Ig conjugates, the binding of Leu2a is completely blocked. When the cells are first incubated with Leu2a followed by its conjugate, and then treated with FK18 and its conjugate, all positive cells are double-stained.

Subsets defined by two surface-antigens

Mononuclear cells were further defined by the presence of two surface-antigens by using membrane-markers in the following combinations: B73.1 and Leu7, B73.1 and FK18, B73.1 and HLA-DR, Leu3a and Leu7, Leu2a and Leu7. In this manner a number of subsets of cells were determined: $CD16^+Leu7^+$, $CD16^+Leu7^-$, $CD16^+CD8^+$, $CD16^+DR^+$, $CD8^+Leu7^+$, $CD8^+Leu7^-$ and $CD4^+Leu^+$. The number of T cells reacting with Leu7 (T^+Leu7^+) was obtained by adding the $CD4^+Leu7^+$ to the $CD8^+Leu7^+$ subset.

Statistics

Because of the sometimes marked asymmetry in the distribution of the findings, the Wilcoxon two-sample rank sum test was used for statistical evaluation. The given *P*-values are uncorrected for the number of comparisons. Correlations were demonstrated by using Spearman's correlation test.

RESULTS

Mononuclear cells

The quantification of leucocytes, lymphocytes, mononuclear cells, null cells, T cells and T-cell subsets, NK cells and NK-cell subsets are given in Tables 2 and 3 and schematically represented in Fig. 1.

NK cells

The number of NK cells, defined as cells reacting with the monoclonal antibody B73.1 (CD16), is strikingly increased in the aged group. This is mostly due to the $CD16^+Leu7^+$ subset (see Fig. 1). Null cells, defined as non-T, non-B, non-monocyte cells also show an increase in the aged, as found in our previous study (Ligthart *et al.*, 1985). In fact, the $CD16^+$ cells nearly account for the entire null cell compartment as proved by the lack of overlap with the T-cell compartment (Table 3). No change is demonstrated in the small $CD16^+CD8^+$ subset; the small number of $CD16^+DR^+$ cells, possibly activated NK cells (Phillips *et al.*, 1984), does not change either.

Leu7 cells

$Leu7^+$ cells are more than doubled in number in the aged and are contained in the subsets $CD16^+Leu7^+$, $CD4^+Leu7^+$ and $CD8^+Leu7^+$. These subsets are all three significantly increased. The proportion of T cells reacting with Leu7, i.e. T^+Leu7^+ is increased.

Table 2. Subpopulations of mononuclear cells (MNC) in young and aged individuals in percentages of mononuclear cells and in absolute numbers

Subpopulation	Young ($n=35$)	Old ($n=33$)	P*
Leucocytes ($\times 10^9$/l)	5·5±1·2†	5·4±1·2	NS
Mononuclear cells	2·20±0·45	2·10±0·48	NS
Lymphocytes	1·76±0·40	1·72±0·44	NS
Null cells (% of MNC)	11±5	17±8	0·001
($\times 10^9$/l)	0·24±0·12	0·36±0·21	0·01
$CD16^+$(B73.1$^+$)	11±5	18±7	0·0001
	0·25±0·10	0·38±0·19	0·0005
$Leu7^+$	10±4	20±9	0·0001
	0·22±0·10	0·43±0·25	0·0002
$CD3^+$(Leu4$^+$)	54±7	49±8	0·01
	1·19±0·31	1·04±0·30	NS
$CD4^+$(Leu3a$^+$)	35±7	34±7	NS
	0·78±0·24	0·71±0·22	NS
$CD8^+$(Leu2a$^+$)	21±5	18±6	0·002
	0·47±0·14	0·37±0·18	0·002
CD4/CD8 (ratio)	1·8±0·5	2·3±1·6	NS

* = Wilcoxon two sample test; NS = not significant.
† = Mean ± 1SD.

Table 3. Natural killer and T-cell subsets expressed as percentage of mononuclear cells and in absolute cell numbers

Subset	Young ($n=35$)	Old ($n=33$)	P*
$CD16^+Leu7^+$ (% of MNC)	5±2†	9±6	0·0001
($\times 10^9$/l)	0·10±0·06	0·20±0·16	0·0005
$CD16^+Leu7^-$	7±3	9±4	0·003
	0·14±0·07	0·18±0·07	0·04
$CD16^+CD8^+$	1±1	1±1	NS
	0·02±0·02	0·02±0·02	NS
$CD16^+DR$‡	2±1	2±2	NS
	0·04±0·02	0·05±0·04	0·04
T^+Leu7^+	4±2	10±8	0·001
	0·09±0·05	0·21±0·18	0·03
$CD8^+Leu7^+$	4±3	8±6	0·002
	0·10±0·06	0·17±0·15	0·03
$CD8^+Leu7^-$	17±5	10±4	0·0001
	0·37±0·12	0·20±0·10	0·0001
$CD4^+Leu7^+$§	0·3±0·4	2±2	0·003
	0·01±0·01	0·03±0·03	0·002

The different numbers of persons tested is not due to selection but to determinations started later in the study.
* = Wilcoxon two sample test; NS = not significant.
† Mean ± 1 SD.
‡ Young $n=7$; old $n=10$.
§ = Young $n=21$; old $n=27$.

T cells

The percentage of T cells is slightly decreased, but not the absolute number. T helper/inducer cells show no change. The number of T suppressor/cytotoxic cells is significantly decreased, and this decrease is confined to the $CD8^+Leu7^-$ subset. The $CD8^+Leu7^+$ subset on the contrary is increased.

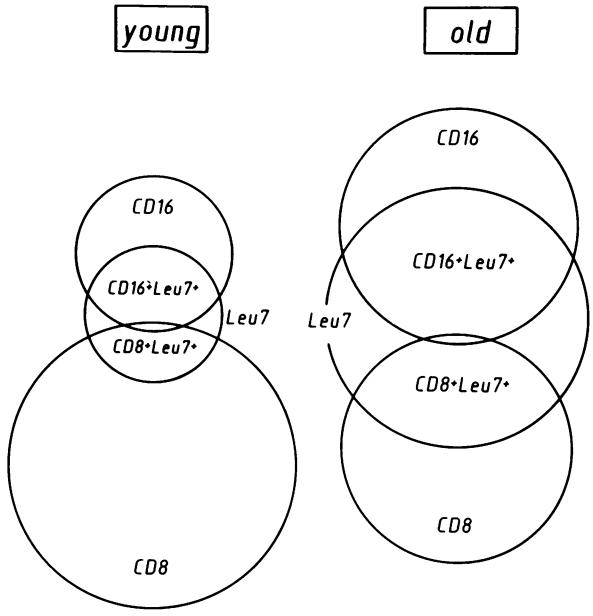

Figure 1. Schematic proportional representation of the main CD16 and CD8 subsets as defined by double-labelling with monoclonal antibodies.

Correlations between T-cell and NK-cell subsets

Spearman correlation analysis shows a significant correlation between the numbers of $CD16^+Leu7^+$ cells, and the T cells bearing the Leu7 marker, i.e. $CD4^+Leu7^+$, $CD8^+Leu7^+$ and T^+Leu7^+ with correlation coefficients of, respectively, 0·32, 0·27 and 0·32 and a P-value of 0·03. The numbers of $CD4^+Leu7^+$ cells and $CD8^+Leu7^+$ are correlated with a coefficient of 0·49, $P=0·001$.

Differences between sexes

All the variables were tested for differences between the sexes. Only the total number of $Leu7^+$ cells was significantly higher in the group of aged men. The number of $Leu7^+$ cells was $0·36 \pm 0·25 \times 10^9/l$ for the aged females and $0·53 \pm 0·23 \times 10^9/l$ for the males ($P=0·02$).

DISCUSSION

Recently we demonstrated that the number of null cells, phenotypically defined as non-T, non-B, non-monocyte cells is strikingly increased in the peripheral blood of optimally healthy aged individuals (Ligthart *et al.*, 1985) who fulfill the stringent admission criteria to immunological studies described in the SENIEUR protocol (Ligthart *et al.*, 1984). This protocol is designed to exclude individuals whose immune system is influenced by the presence of disease or the use of medication. The application of such a protocol is a prerequisite if one wishes to study ageing as such.

NK cells

Reactivity of practically all null cells with the B73.1 (CD16) monoclonal antibody indicates that these cells are NK cells. The increased number of peripheral blood NK cells in the optimally healthy aged as compared to the young can have several meanings.

1. It could be a consequence of positive selection, the most healthy individuals having the highest number of NK cells.
2. A higher demand for NK cells could be caused by an increased strain on the immune system in the aged, for example by increased occurrence of viral infections or malignant transformation.
3. An increase in the number of NK cells could be an attempt of the immune system to compensate for a defective T-cell system.
4. It could be a numerical compensation for a decreased function of the individual NK cell.
5. It could be a primary age-related defect of regulation or proliferation.
6. Finally, it could be due to an age-related change in compartmentalization and be the result of a shift of NK cells towards the intravascular space.

An answer to these questions might be given by studying the NK cell function at the level of the individual cell simultaneously with T-cell function, in the young and aged, in health and disease.

The increase in the number of NK cells demonstrated in these optimally healthy aged could mean that the NK function is increased in this age group and this might be an advantage for survival. Some studies of NK function in aged people not selected with use of the SENIEUR protocol suggest an increase in NK function (Onsrud, 1981; Batory *et al.*, 1981; Pross & Baines, 1982). These findings could well be related to the increased number of NK cells found in our study.

NK cells defined as $CD16^+$ cells do not bear the T-cell marker CD3 (Perussia *et al.*, 1983; Lanier *et al.*, 1983) and their genome has not undergone a rearrangement for the beta chain of the T-cell receptor (Lanier *et al.*, 1986), which makes them fundamentally distinct from T lymphocytes. It follows that the small subset of $CD16^+CD8^+$ cells is $CD16^+CD8^+CD3^-$. Within this small subset some cells are probably $CD16^+CD8^+Leu7^+$. This is supported by our recent description of a patient with a non-malignant expansion of large granular lymphocytes (LGL) bearing these three markers (Van de Griend *et al.*, 1985).

T cells

Within the T-cell subset, the $CD8^+$ suppressor/cytotoxic subset was significantly decreased, as shown before (Nagel *et al.*, 1983; Ligthart *et al.*, 1985). The present study demonstrates that this decrease is due to a decrease of the $CD8^+Leu7^-$ subset, while the $CD8^+Leu7^+$ subset is increased: in the older age-group nearly half of the $CD8^+$ cells are also $Leu7^+$. An increase of this $CD8^+Leu7^+$ compartment has also been observed under conditions of depressed immune function such as during repopulation after bone-marrow transplantation (Favrot *et al.*, 1983), cytomegalovirus infection (Würsch *et al.*, 1985), and in AIDS (Lewis *et al.*, 1985). According to Phillips & Lanier (1986) $CD8^+Leu7^+$ cells mediate lectin-dependent and anti-CD3 induced cytotoxicity, and could be *in vivo*-primed CTL. The increased number of these cells in the aged could be an advantage for survival, or just a result of increased occurrence of viral infections. Functional testing of the cytotoxicity of these cells in an aged group could give some insight. The increase of the NK and T-cell subsets reacting with Leu7 ($CD16^+Leu7^+$, $CD4^+Leu7^+$ and $CD8^+Leu7^+$) were all significantly correlated. The role of Leu7

on the lymphocyte membrane needs further study to allow interpretation of these findings.

A difference between the sexes was only noted for the number of Leu7$^+$ cells, which were increased in the aged males. This has also been found in males aged up to 70 years (Abo, Cooper & Balch, 1982). One explanation can be that males are biologically older then their calendar-matched female counterparts.

In conclusion this study indicates a striking increase in the number of NK cells defined as B73.1$^+$ (CD16) cells in peripheral blood in ageing. T suppressor/cytotoxic cells of the subset CD8$^+$Leu7$^-$ are decreased. All NK cell and T-cell subsets bearing the Leu7 antigen, namely CD16$^+$Leu7$^+$, CD4$^+$Leu7$^+$ and CD8$^+$Leu7$^+$ are increased. The significance of this study is four-fold.

1. These findings can be a manifestation of a deficiency of the senescent immune system, or on the contrary be due to positive selection, and characterize the optimal state of the immune system in the healthy aged. In both cases these changes may be related to survival and be a measure for the ageing of the immune system.

2. This study provides age-related reference-values determined in optimally healthy subjects fulfilling the admission criteria of the SENIEUR protocol. These reference values are important in the study of ageing of non-optimally healthy persons and are essential for the assessment of the influence of ageing versus the influences of disease on the immune system.

3. These findings also have consequences for the study of the immune system in disease, because it stresses the importance of the use of age-related reference values, or age-matched control groups in such studies.

4. If these changes are the origin of an immunological defect in ageing, efforts to reconstitute the immune system could be based on this finding.

ACKNOWLEDGMENTS

We thank Dr G. Trinchieri and Dr F. Koning for the kind gifts of B73.1 and FK18 monoclonal antibodies, Dr T. Stijnen for his statistical advice, Mrs A. Velthuis for the preparing of the manuscript and Dr J. H. F. Falkenburg, J. Krebbers and Dr. E. L. Lagaay for invaluable support.

This study was supported in part by the Foundation for Medical Research FUNGO, The Hague (grant no. 13-40-76), by the Steering Committee for Ageing Research SOOM, Nijmegen (grant no. 83-2-45) and by the Netherlands Organization for Applied Research TNO, The Hague, The Netherlands and effectuated within the framework of 'EURAGE', the Concerted Action Programme on Ageing of the European Economic Community.

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