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Subpopulations of mononuclear cells in ageing: expansion of the null cell compartment and decrease in the number of T and B cells in human blood

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Summary. Study of the immune system in ageing has yielded conflicting results. These controversies are mainly due to the selection of the subjects studied. We investigated the mononuclear cell subpopulations in the peripheral blood of subjects fulfilling strict admission criteria meant to exclude persons with diseases that influence the immune system. These criteria are described in the SENIEUR protocol devised by a working group in the framework of EURAGE, the Concerted Action Programme on Ageing of the European Community. We compared two groups of volunteers aged 25–34 years, and 75–84 years. Mononuclear cells were investigated by two-wavelength immunofluorescence combined with phase-contrast microscopy. We found a striking increase in the number of 'null' cells (non-T, non-B, non-monocyte) in the blood of the aged persons. The number of T cells was decreased, especially in the suppressor/cytotoxic subset. The number of B cells was slightly, but significantly, decreased; the number of monocytes did not change. The changes in these cell populations may be related to functional changes, and their quantification could be used to monitor attempts to reconstitute the immune defects in ageing. These findings can also serve as reference values in the study of aged persons not fulfilling the SENIEUR criteria, which, in turn, can contribute to the dissection of the influence of disease versus age on the immune system.

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INTRODUCTION

In human immunogerontology, the peripheral blood has been studied in depth to gain insight into the cause of the immunodeficiency of ageing. However, these studies have led to a great number of often contradictory results: the total number of T cells has been reported to increase or decrease with ageing (Hallgren *et al.*, 1978; Hallgren, Jackola & O'Leary, 1983; Nagel *et al.*, 1983). T-helper cells were found to increase or decrease (Van de Griend *et al.*, 1982; Mascart-Lemone *et al.*, 1982). The T-suppressor cells seemed to increase or decrease (Ceuppens & Goodwin, 1982; Nagel *et al.*, 1983). The total number of B cells showed a decrease or no change (Gupta & Good, 1979; Cobleigh, Braun & Harris, 1980). The monocytes showed no change (Van de Griend *et al.*, 1982). Null cells, also called third population cells or L cells (see Horwitz & Bakke, 1984), containing most of the natural killer cell activity, have, to our knowledge, never been evaluated in ageing—and their existence has even been denied (Pepys, Tennent & Pepys, 1981). Cells reacting with the HNK-1 (now Leu 7) monoclonal antibody, and thought to be associated with killer and natural killer function (Abo & Balch, 1981) partly contained in the null-cell compartment, were reported to increase with age (Abo, Cooper & Balch, 1982b).

In our view, the main reason for these divergent results is the heterogeneity of the subjects admitted to immunogerontological studies. The presence of unrecognized disease is frequent in the elderly and can influence the findings. Therefore, we selected the subjects for our studies by using the strict admission criteria of the SENIEUR protocol (Ligthart *et al.*, 1984) elaborated in the framework of EURAGE, the Concerted Action Programme on Ageing of the European Economic Community.

We report here our studies of the haematological parameters and the subpopulations of mononuclear cells of the human peripheral blood in ageing. We used two-wavelength immunofluorescence combined with phase-contrast microscopy. We found a significant decrease in the number of T cells, being most pronounced in the suppressor/cytotoxic (Leu $2a^+$) subset, and a slight but statistically significant decrease in the number of B cells. The number of monocytes did not change. The most striking finding was a considerable increase in the number of null cells (non-T, non-B, non-monocyte) in the older age group.

MATERIALS AND METHODS

Persons studied

Twenty-four volunteers (12 males and 12 females) aged 75–84 years (mean: 81 years) and 24 volunteers (12 males and 12 females) aged 25–34 years (mean: 28 years), all of Dutch caucasoid origin, were admitted to the study. An additional 20 young and 8 old subjects were examined only for the T-cell subsets. 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). Briefly, this protocol details exclusion criteria based on clinical information, laboratory data with an age-dependent reference range, and sets rules for the limitation of pharmacological interference. Excluded are: infection; inflammation; malignancy, past and present; other overt disease and all conditions known to influence the immune system, such as cardiac insufficiency, malnutrition, or—in the young comparison group—pregnancy. The laboratory tests leading to exclusion are interpreted wherever possible with age-dependent reference values and include a haematological screening, hepatic and renal function tests and a urinalysis. Also excluded are subjects taking medication with a known influence on the immune system and those using any medication prescribed for the treatment of a defined disorder.

Haematological investigations

The erythrocyte sedimentation rate (ESR) was determined in the first hour; the haemoglobin, haematocrit, erythrocytes, cell indices and total number of leucocytes were determined by means of a Coulter counter (Coulter Electronics, Hialeah, FL). The leucocyte differentiation count was performed with a Haemalog D (Technicon Instruments, Tarrytown, N.Y.).

Preparation of mononuclear cells

All samples were processed at less than 1 hr after venepuncture. The preparation and the handling of the cells for immunofluorescence have been described in detail in a previous report (Schuit, Hijmans & Asma, 1980). Coagulation of blood was prevented by 5% (w/v) EDTA (Titriplex III, Merck AG, FRG) in phosphate-buffered saline (PBS), pH 7·2. The blood was diluted with an equal volume of PBS and mononuclear cells were isolated by centrifugation at room temperature (22°) for 13 min at 1000 ***g*** on a leucocyte separation medium (LSM, Litton Bionetics, Kensington, MD) in the ratio 5 ml of blood to 2 ml of LSM. The cells of the interfaces were washed once in PBS containing 5% bovine serum albumin (BSA) (Boseral, Organon Teknika, Oss, The Netherlands) and 0·1% EDTA. Unless stated otherwise, the cells were fixed with freshly prepared 0·04% formaldehyde (acid-free 37%, P.A. Merck, Darmstadt, FRG) in PBS for 10 min at room temperature. They were washed once with 5% BSA and the pellet was resuspended in 1% BSA. In a disposable plastic tube, 0·025 ml of the appropriate antiserum or conjugate dilution was added to 0·05 ml of the cell suspension (about 10^6 cells). This suspension was incubated at room temperature for 30 min and gently shaken every 10 min. The cells were washed once with 1% BSA and centrifuged in the fixed-angle Serofuge (Clay-Adams, Parsippany, NJ). The supernatant was removed as completely as possible. For the second antibody layer, this cycle was repeated. The cell pellet was resuspended with a vortex mixer in the remaining fluid and deposited into a small drop of 90% glycerol in PBS, pH 7·8, placed on a cover glass (24 × 32 mm). The suspension was then covered with an object glass without exerting pressure and the cover glass was sealed with paraffin.

Table 1. Conventional and monoclonal antisera

Antibody specificity	Ig class/ subclass	Working dilution*	Fluorochrome	Assigned specificity	Source
T	Horse IgG	40	TRITC	T lymphocytes	†
Fab	Goat F(ab')$_2$	32	FITC	B lymphocytes, monocytes	Nordic Imm. Lab.‡
IgM	Goat F(ab')$_2$	48	FITC	B lymphocytes	Kallestad§
IgD	Goat F(ab')$_2$	24	TRTIC	B lymphocytes	Kallestad
Kappa	Goat F(ab')$_2$	48	FITC	B lymphocytes	Kallestad
Lambda	Goat F(ab')$_2$	40	TRITC	B lymphocytes	Kallestad
Leu 4	Mouse IgG1	20	FITC	T lymphocytes	Bect. Dick.¶
Leu 3a	Mouse IgG1	80	–	T helper/inducer	Bect. Dick
Leu 2a	Mouse IgG1	80	–	T suppressor/cytotoxic	Bect. Dick
HLA-DR	Mouse IgG2a	100	TRITC	B lymphocytes, monocytes, activated T lymphocytes	Bect. Dick.
OKM1	Mouse IgG2b	100	–	Monocytes, granulocytes	Ortho Diagn.**
HNK-1	Mouse IgM	2000	–	NK,K cells	T. Abo††
GAM/IgG1	Goat IgG	64	FITC	Mouse IgG1	Nordic Imm. Lab.‡
GAM/IgM	Goat IgG	64	FITC	Mouse IgM	Nordic Imm. Lab
GAM/mIg‡‡	Goat IgG	32	FITC	Mouse Ig	Nordic Imm. Lab.

* Reciprocal value.
† Locally prepared by adsorption of commercial anti-lymphocyte serum.
‡ Nordic Immunological Laboratories, Tilburg, The Netherlands.
§ Kallestad, Chaska, MN.
¶ Becton Dickinson, Mountain View, CA.
** Ortho Diagnostics, Raritan, NJ.
†† Dr T. Abo, University of Alabama, Birmingham, AL.
‡‡ Adsorbed with human immunoglobulin.

Antisera and detection of subpopulations

The antisera and fluorescent conjugates used, their classes, subclasses and working dilutions are given in Table 1. The specificity and the working dilutions of the antisera used were tested as previously described (Schuit, Morée van der Linde & Hijmans, 1981).

Null cells

The null cells defined as non-T, non-B, non-monocyte were determined in three ways:

(i) the number of cells negative for both a conventional anti-T marker and an anti-Fab antiserum;

(ii) the number of cells negative for both anti-Leu 4 and anti-HLA-DR;

(iii) calculation of the null cell compartment by subtracting from the total number of mononuclear cells the number of anti-Leu 4 positive cells, the anti-IgM, anti-IgD positive cells and the monocytes as recognized by morphology in phase-contrast microscopy (for morphologic criteria see below). The monoclonal antibody HNK-1, kindly provided by Dr T. Abo, University of Alabama, Birmingham, AL (Abo & Balch, 1981), was used in an attempt to detect killer and natural killer cells.

T cells

T cells were enumerated with anti-Leu 4 FITC and also with a TRITC-labelled IgG fraction of a horse anti-human T-cell antiserum. T-helper/inducer and T-suppressor/cytotoxic cells were enumerated by using anti-Leu 3a and anti-Leu 2a, respectively. Activated T cells were identified by their simultaneous staining with anti-Leu 4 FITC and anti-HLA-DR TRITC in the two-wavelength immunofluorescence method.

B cells

B cells were enumerated in four different ways: (i) with GAHu/Fab FITC; (ii) with a mixture of specific anti-IgM FITC and anti-IgD TRITC; (iii) with a mixture of anti-kappa FITC and anti-lambda TRITC, and (iv) as HLA-DR TRITC-positive lymphocytes, excluding activated T cells with anti-Leu 4 FITC.

Monocytes

Monocytes were defined by morphology in phase-contrast microscopy according to previously described morphological criteria (Schuit & Hijmans, 1980), mostly in association with the fluorescence staining with anti-Fab, HLA-DR or OKM1.

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 screened by hand and each cell was examined individually for its immunological reactivity by narrow-band excitation 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. Each subset was expressed as a percentage of total mononuclear cells. This percentage was multiplied by the absolute number of mononuclear cells to obtain the absolute number of cells in a subset.

Statistics

Because of small samples and 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.

RESULTS

Haematological findings

The haematological findings are given in Table 2. The erythrocyte sedimentation rate (ESR) showed a slight increase in the aged group. These values are not to be considered as reference values for these age groups, but may apply to a highly selected group of 'optimally healthy' subjects from an immunological point of view, who satisfy the criteria of the SENIEUR protocol which used age-related limits for ESR and haemoglobin as admission criteria.

Immunological findings

Null cells

The quantification of the null cells defined as non-T, non-B, non-monocyte is given in Table 3. There was a striking increase in the null cells in the aged group, both in percentages of total mononuclear cells and in absolute numbers. Cells positive for HNK-1 (anti-Leu 7) were markedly increased in the aged group.

T cells

The quantification of the T-cell compartment is presented in Table 4. In the aged persons, there was a decrease in the number of T cells as defined with several pan-T markers. The decrease in anti-Leu 3a positive (T-helper/inducer) cells was less marked than the decrease in anti-Leu 2a positive (suppressor/cytotoxic) cells; consequently, the Leu 3a/Leu 2a (helper/

Table 2. Haematological findings

Variable and units	Young ($n=24$)	Old ($n=24$)	P*
ESR (Westergren, mm)	4±3†	10±6	0·001
Haemoglobin (mmol/l)	9·4±0·4	9·2±0·6	NS
Haematocrit	0·45±0·03	0·44±0·03	NS
Erythrocytes ($\times 10^{12}$/l)	5·05±0·48	5·00±0·46	NS
MCV‡ (fl)	89±4	88±5	NS
Leucocytes ($\times 10^9$/l)	6·2±1·6	5·8±1·4	NS
Mononuclear cells ($\times 10^9$/l)	2·25±0·53	2·06±0·55	NS
Lymphocytes ($\times 10^9$/l)	1·79±0·47	1·62±0·48	NS
Monocytes ($\times 10^9$/l)	0·46±0·16	0·44±0·21	NS
Thrombocytes ($\times 10^9$/l)	239±64	208±41	NS

* Wilcoxon two-sample test; NS, not significant.
† Mean ±1 SD.
‡ Mean corpuscular volume.

Table 3. Null cells expressed as percentages of mononuclear cells and in absolute cell numbers

Determination	Young ($n=24$)	Old ($n=24$)	P*
Null cells			
Leu 4^-DR^- (%)	11±4†	18±8	0·0001
($\times 10^9$/l)	0·24±0·09	0·37±0·17	0·002
Leu 4^-B^- monocyte$^-$	11±5	16±6	0·001
	0·24±0·12	0·34±0·15	0·02
T^-Fab^- monocyte$^-$	9±2	13±5	0·003
	0·19±0·06	0·26±0·13	NS
Natural killer and killer cells			
HNK	11±6	21±10	0·0004
	0·24±0·16	0·43±0·26	0·005

* Wilcoxon two-sample test.
† Mean ±1 SD.

Table 4. T cells expressed in percentages of total mononuclear cells and in absolute cell numbers

Antibody	Young ($n=44$)	Old ($n=32$)	P*
Anti-T (polyclonal)† (%)	58±7‡	50±9	0·005
($\times 10^9$/l)	1·31±0·40	1·05±0·31	0·02
Leu 4 (pan-T)	54±8	47±8	0·001
	1·20±0·37	0·95±0·27	0·006
Leu 3a (helper/inducer)	35±7	32±9	NS
	0·80±0·26	0·67±0·24	0·02
Leu 2a (suppressor/cytotoxic)	21±5	17±5	0·001
	0·47±0·16	0·35±0·13	0·001
Leu 4^+DR^+ (activated T)†	1±1	2±2	NS
	0·03±0·03	0·04±0·03	NS
Leu 3a/Leu 2a (ratio)	1·8±0·5	2·1±0·8	NS

* Wilcoxon two-sample test; NS, not significant.
† Young $n=24$; old $n=24$.
‡ Mean ±1 SD.

suppressor) ratio showed a slight but non-significant increase. Anti-Leu 4 anti-HLA-DR positive cells, thought to be activated T cells, showed no difference between the young and the old group.

B cells

The number of B cells given in Table 5 was slightly, but significantly, decreased in the aged group; this was most clear when these cells were defined by antisera against IgM and IgD.

Monocytes

The number of monocytes was remarkably constant in the two age groups. With phase-contrast microscopy, we found $0{\cdot}61\pm0{\cdot}15\times10^9$ cells/l in the young group and 0·64±0·23 in the aged. This represented 25±5% of mononuclear cells in the young and 31±7% in the aged. The number of cells reacting with the monoclonal antibody OKM1 is slightly larger because some lymphocytes are also positive. These values were $0{\cdot}69\pm0{\cdot}22\times10^9$ cells/l for the young versus $0{\cdot}68\pm0{\cdot}27\times10^9$ cells/l for the aged.

Table 5. B cells expressed as percentages of mononuclear cells and in absolute cell numbers

Antibody	Young ($n=24$)	Old ($n=24$)	P*
Anti-IgM and Anti-IgD (%)	6·8±1·9†	5·4±2·6	0·02
($\times 10^9$/l)	0·15±0·04	0·11±0·07	0·006
Anti-kappa and anti-lambda	6·2±2·0	4·9±2·1	0·03
	0·14±0·04	0·10±0·06	0·005
Anti-Fab	5·3±2·5	5·2±2·6	NS
	0·12±0·07	0·10±0·05	NS
Leu 4^-DR^- lymphocytes	5·6±2·1	4·7±2·4	NS
	0·12±0·04	0·10±0·06	0·04

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

DISCUSSION

In this study, the composition of the mononuclear cell compartment of human peripheral blood in ageing was analysed. Both young and aged subjects met the strict admission criteria of the SENIEUR protocol (Ligthart *et al.*, 1984) and are, therefore, to be considered as 'optimally healthy' from an immunological point of view. This permits a closer analysis of ageing and its effect on the immune system. We believe that this is the reason why we can provide new information and why our findings only partly confirm those of former studies.

The most striking new finding in this study is the increase of approximately 50% in the number of null cells, defined as non-T, non-B, non-monocyte. This compartment contains precursor T and precursor B cells (Gathings, Kubagawa & Cooper, 1981) and cells possessing killer (K) or natural killer (NK) function. The expansion of the null cell compartment could be due to any of these cell types.

A first hypothesis is that the increase in null cells is related to the decrease in T cells, which is generally considered to result from the involution of the thymus with ageing. T-cell precursors could persist in the peripheral blood as 'pre-thymic' cells which still lack the mature T-cell antigens recognized by most pan-T markers and, thus, be assessed as null cells. Several findings support this view. In ageing, there is an increase of cells which carry a helper/inducer or a suppressor/cytotoxic subset marker while lacking a pan-T cell marker such as OKT 3 or Leu 4 (Hallgren *et al.*, 1983), a phenotype thought to characterize immature T cells (Reinherz *et al.*, 1980). The lactate dehydrogenase (LDH) isoenzyme pattern of the rosetting cells indicates immaturity and resembles that found after thymectomy (Van de Griend *et al.*, 1982). However, other markers of immature T cells such as OKT6, OKT9 and OKT10 (Nagel *et al.*, 1983; Van de Griend *et al.*, 1982) are not increased in ageing.

A second possibility is that the expansion of the null cell compartment is caused by an increase of NK cells. This possibility is not contradicted by the absence of an increase of OKM1-positive cells in the aged group because OKM1 reacts strongly with monocytes but not, or at much lower density, with NK cells (Trinchieri & Perussia, 1984; Horwitz & Bakke, 1984). Cells reacting with the monoclonal antibody HNK-1, now Leu 7, thought to be killer or natural killer cells (Abo & Balch, 1981), almost double in numbers in the aged group in our study (Table 3). However, HNK-1 does not react only with null cells but also with a considerable number of T cells (Abo, Cooper & Balch, 1982a) and therefore is not to be considered a null cell marker. From the same study, it is known that the NK function is low in the HNK^+T3^+ subset, and is mostly confined to the HNK^+T3^-, that is, the HNK^+ null compartment. From our data, it is not possible to determine which of these subsets change with ageing, and further studies are obviously needed. The lineage of the NK cell is not yet defined. This cell could be of monocytic, T cell or separate lineage. It is not excluded that a T-cell precursor is the effector cell of NK function, but we did not find a correlation between the decrease in the number of T cells and the increase of the null cells.

The possibility that the increase in null cells is due to precursor B cells is unlikely because the latter would have been positive for HLA-DR (Gathings *et al.*, 1981). A monocytic origin of the null cell is unlikely because the increased cell population is negative for HLA-DR antigens and for anti-Fab, and a large majority does not react with OKM1.

The significance of the decrease in the number of B cells remains unclear. Serum immunoglobulin levels do not decrease in ageing. IgG and IgA levels even increase (Kalff, 1970). This could be a result of decreased catabolism of immunoglobulins, an increase in the number of secreting plasma cells in the bone marrow, or the amount of immunoglobulin secreted per plasma cell could be increased (Jerne, 1984). The decrease in B cells in the peripheral blood and an increase of end cells in the bone marrow could be due to a change in maturation, proliferation or homing of the B cell in ageing. The study of subpopulations of B cells according to multiple isotypes on the cell membrane (Vessière-Louveaux, Hijmans & Schuit, 1981) could give further insight.

In conclusion, our findings indicate that ageing in man is accompanied by changes in several mononuclear cell compartments in human peripheral blood. The significance of this study is three-fold.

(i) The reference values obtained from the subjects in this study, fulfilling the SENIEUR criteria in the age range of 75–84 years, are useful in the study of the non-healthy aged to assess the influence of ageing versus the influence of disease on the immune system.

(ii) The increase in null cells is of significance to the understanding of the changes in the immune system in ageing. In a negative way, it could be an expression of deficiency; in a positive way, an advantage for survival. In both cases, the number of null cells

could be a measure for the ageing of the immune system.

(iii) If the changes in the null cell compartment are the origin of an immunological defect in ageing, efforts to reconstitute the immune system could be based on this finding.

Further phenotypic and functional analysis of the null cell population in human blood is necessary for the understanding of the changes in the immune system in ageing.

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