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Two-Color Immunofluorescene and Flow Cytometric Analysis of Peripheral Blood Lymphocyte Subsets in Aging Humans

Masahiro Senju, Kazuya Makiyama, Hidetosi Oda, Koji Nakamuta, Katsuhisa Omagari, Minoru Itsuno and Kohei Hara

The Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan

Summary: Using two-color immunofluorescence, multiparameter flow cytometry has been used to examine the antigenic characteristics of the nature of changes due to aging in lymphocyte subests. The percentage and absolute numbers of T cells (CD3+ T) declined in the aging humans. This decline was associated with a decreasing trend of CD8+ T (cytotoxic/suppressor) cells in aging. The aged men had a slight but non-significant increased proportion of CD4+Leu8- T cells, which mediate the helper functions in B cell differentiation in association with an increasing tendency in the number of CD4+ T cells. A wide variety of changes took place in activated T cells expressing antigens such as Leu17 (CD38), HLA-DR, or HLA-DQ. Although the decrease in the number of total CD8+ T cells was only slight, the proportion of CD8+ cell bearing Leu7 (CD57) antigen was strikingly increased in the elderly. For NK cells, a total number of NK cells (Leu19+CD3-) in the aged subjects was increased, but a subpopulation of cells having the high NK activity (Leu19+CD57- cells) decreased in the elderly men. The present study revealed various changes in lymphocyte subsets in aging humans.

Key Words: lymphocyte subsets; aging; flow cytometry

Introduction

Recent progress in monoclonal antibodies to cell surface antigens has allowed detailed characterization of subpopulations of lymphoid cells.[1] Flow cytometry utilizes rapid automated counting of large numbers of cells, which permits multiple surface markers to be analysed. Flow cytometry can also provide a quantitative measurement of intensity which may be related to receptor density. The use of simultaneous two-color immunofluorescence has added a new dimension to this type of analysis. Furthermore, the use of a whole blood assay avoids the use of density gradient centrifugation, which might potentially alter the ratios of lymphocyte subsets.[2,3]

The aim of the present study was to investigate the influence of aging on the distribution of a wide variety of human peripheral blood lymphocyte subpopulations. Two-color immunofluorescence staining by the use of a whole blood assay and four parameters (2 surface antigens, cell size and granularity) flow cytometry were used.

Reprint request to: M. Senju, M D,
The Second Department of Internal Medicine, Nagasaki University School of Medicice, 7-1 Sakamoto-machi, Nagasaki 852, JAPAN

Materials and Methods

1. Study population

Peripheral blood was obtained from 42 healthy Japanese. Of the 42, 18 volunteers, 9 men and 9 women, were the younger group aged 23-35 years (mean; 30 ± 3). Eight volunteers, 4 men and 4 women, were the middle-aged group aged 36-57 years (mean; 46 ± 8). And 16 volunteers, 5 men and 11 women, were the older group aged 66-80 years (mean; 71 ± 4). Subjects of 65 years of age and over were selected from those who presented themselves for a health check at the Nagasaki City Medical Association Hospital. These subjects met the study criteria below; the absence of disease for at least 3 years prior to the study entry and no abnormal data on blood pressure, white blood cell count (WBC, RBC, Hgb, and Ht), or urinalysis (glucose, protein, and occult blood).

2. Monoclonal antibodies

Eleven combinations of monoclonal antibodies conjugated with FITC and PE were used. All monoclonal antibodies were obtained from the Becton Dickinson Monoclonal Center, Inc. (Mountain View, C. A.) The specificities of these reagents used in this study are presented in Table 1. They were all directly conjugated with FITC or PE.

3. Two-color direct immunofluorescence staining of peripheral blood lymphocytes

Whole blood collected by venipuncture with EDTA was drawn between 8 and 12 AM to exclude the influence of circadian variation of lymphocyte subpopulations.[4] A

Table 1. Specificity of monoclonal antibodies

Monoclonal antibodies (FITC/PE)	Antigen cluster designation	Target subset
HLe-1/Leu-M3	CD45/CD14	For electronic gate setting for lymphocytes
IgG1/IgG2	-/-	For negative control and non-specific binding
Leu-3/Leu-8	CD4/-	Suppressor-inducer and helper-inducer T cells
Leu-4/IL2-R	CD3/CD25	Activated T cells
Transferrin-R/Leu-4	CD71/CD3	Activated T cells
Leu-4/Leu-17	CD3/CD38	Activated T cells
Leu-4/HLA-DR	CD3/-	Activated T cells
HLA-DQ/Leu-4	-/CD3	Activated T cells
Leu-7/Leu-2	CD57/CD8	Leu-7+ cells
Leu-4/Leu-19	CD3/CD56	NK cells and CTL
Leu-7/Leu-19	CD57/CD56	NK cell subsets

FITC, fluorescein isothiocyanate; PE, phycoerythrin
NK cells, natural killer cells; CTL, cytotoxic T lymphocytes

whole blood assay, as previously described,[2,3] was used so as to avoid the use of density gradient centrifugation which might potentially alter the ratio of lymphocyte subsets. Briefly, 100 μl of anticoagulated whole blood was placed into a 12x75 mm polystyrene tube (Falcon Plastics, Oxnard, C. A.). 20u μl of each monoclonal reagent was added to each tube. After incubation for 15 min at room temperature, 2 ml of FACS Lysing Solution (Becton Dickinson) were added to each tube. The samples were then agitated and incubated for 10 minutes. The samples were washed once in Dulbecco's Phosphate Buffered Saline (PBS) without calcium and magnesium (Gibco, Grand Island, N. Y.) containing 0.1% sodium azide. The cell sediment was resuspended in 0.5 ml of 0.5% paraformaldehyde in PBS.

4. Two-color flow cytometry[2,3]

Analysis by two-color flow cytometry was performed with a FACScan (Becton Dickinson, FACS Division, Sunnyvale San, C. A.). A Consort 30 computer system (Becton Dickinson, FACS Division) was used for the flow cytometric data acquisition and analysis. Thirty thousand cells were acquired in list mode for each sample for two-color analysis. The optimum peripheral blood lymphocytes gate for an individual subject was set, using a LeucoGATE (Becton Deckinson), on the cytogram of forward light scatter (FSC) which is related to cell size and of side light scatter (SSC) which provides a measure of cell granularity. Subsequent samples for the same subject were analysed using this gate. A Simultest Control reagent (IgG1 FITC + IgG2 PE, Becton Dickinson) was used to set the optimum fluorescence quadrant markers for each subject and to monitor non-specific staining. Percentages were calculated based on the number of lymphocytes found in each quadrant. Inter-assay reproducibility was checked using Cali-BRITE beads (Becton Dickinson) and AutoCOMP software (Becton Dickinson).

A complete blood count including automated differential was simultaneously performed on each sample. Absolute counts for lymphocyte subsets were derived using the white blood cell count, the lymphocyte fraction from the complete blood count and the proportion of antibody positivity from the FACScan.

5. Statistical methods

Statistical analysis of results was performed by using Student's paired or unpaired t test, and a probability of equal to or less than 5% was considered to be significant.

Results

There was no significant difference in the proportion of T cells (CD3+ T) between the younger and middle-aged groups, and so is the case with B cells (HLA-DR+CD3-). In the older group compared with the other two groups T cells tended to decrease, whereas B cells remained unchanged. With aging the percentage and absolute numbers of NK cells (Leu19+CD3-) tended to increase (Table 2, Fig. 1).

Subpopulations of T cells investigated were CD4+ T

Table 2. Proportions of T lymphocytes, B lymphocytes, and NK cells in peripheral blood

Group	WBC	Lymphocytes	T cells	B cells	NK cells
Young			66.4 ± 7.4	13.7 ± 3.9	12.8 ± 7.7
	5539 ± 1264	1890 ± 604	1246 ± 401	264 ± 115	257 ± 247
Middle-aged			65.9 ± 5.3	12.7 ± 3.3	17.4 ± 5.2
	5238 ± 927	1769 ± 599	1177 ± 432	226 ± 93	303 ± 112
Aged			59.8 ± 14.3	13.3 ± 4.2	21.8 ± 13.3*
	5600 ± 1557	1963 ± 819	1196 ± 647	271 ± 174	393 ± 229

*$p < 0.05$; compared with the young group.
Upper line values, % of total lymphocytes (mean ± SD).
Lower line values, absolute count (/microliter, mean ± SD).

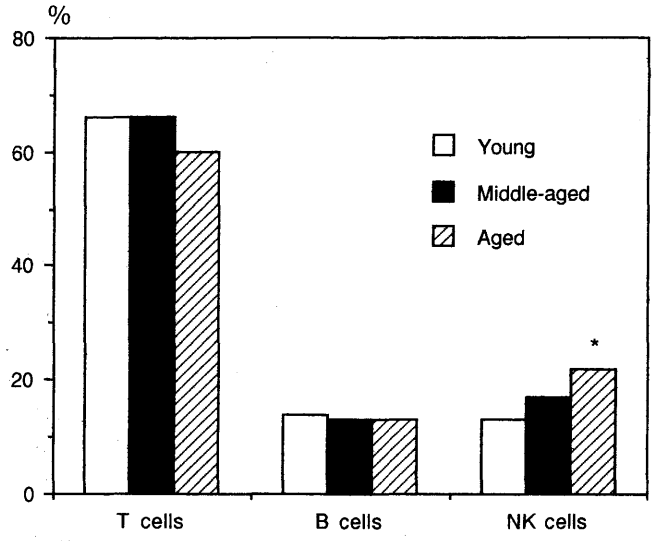

Fig. 1. T lymphocytes, B lymphocytes, and NK cells in peripheral blood (% of total lymphocytes). The proportion of NK cells in the aged subjects was significantly increased compared with that of the young group (*p<0.05).

(helper/inducer) cells and its two components, suppressor-inducer T cells (CD4+Leu8+)[5-7] and helper-inducer T cells (CD4+Leu8-),[5,7,8] and CD8+ T (cytotoxic/suppressor) cells. There was no significant difference in the proportion of CD4+ T cells among the three groups, but CD4+Leu8- T cells tended to increase with advancing age. Although the proportion of CD8+ T cells in the older group was lower than that in the younger group, this difference was not statistically significant (Table 3).

Table 3. Human T lymphocyte subsets in young, middle-aged, and aging subjects

Group	CD4+ cells	CD4+, Leu-8+	CD4+, Leu-8-	CD8+ cells
Young	36.3 ± 5.9	29.4 ± 4.8	6.9 ± 3.3	36.7 ± 7.6
	664 ± 219	535 ± 168	129 ± 70	676 ± 271
Middle-aged	39.1 ± 6.7	31.5 ± 6.0	7.6 ± 2.6	32.6 ± 8.2
	675 ± 265	541 ± 201	135 ± 79	588 ± 276
Aged	36.7 ± 10.2	27.8 ± 9.5	8.9 ± 3.6	33.0 ± 9.7
	717 ± 366	537 ± 287	171 ± 91	654 ± 378

Upper line values, % of total lymphocytes (mean ± SD).
Lower line values, absolute count (/microliter, mean ± SD).

Activated T cells were investigated by using five different surface markers. Activated CD3+ T cells bearing interleukin 2 receptor (CD25), an early activation marker,[9] were not affected by age. As to activated T cells expressing intermediate activation markers such as transferrin receptor (CD71) and CD38 (Leu17),[9] it was found that the percentage of CD38 positive T cells were significantly decreased in the older ($p < 0.001$) and middle-aged groups ($p < 0.05$) compared with that in the younger group. Activated T cells expressing a late activation marker,[9] either HLA-DR or HLA-DQ (Leu10) antigen, tended to increase in the older group (Table 4).

Table 4. Activated T lymphocytes in young, middle-aged, and aging subjects

Group	CD25+ T	CD71+ T	CD38+ T	DR+ T	DQ+ T
Young	13.4 ± 4.5	1.1 ± 0.3	34.3 ± 7.8	14.8 ± 6.9	1.2 ± 0.7
	242 ± 100	20 ± 8	635 ± 218	272 ± 151	23 ± 19
Middle-aged	15.6 ± 5.1	1.2 ± 0.6	27.6 ± 6.2*	17.3 ± 6.1	1.9 ± 1.0*
	269 ± 121	21 ± 8	489 ± 215	323 ± 191	33 ± 19
Aged	13.0 ± 5.7	0.9 ± 0.3	20.8 ± 8.9***	17.6 ± 8.7	2.1 ± 1.4*
	232 ± 89	18 ± 11	409 ± 250**	375 ± 297	44 ± 37*

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; compared with the young group.
Upper line values, % of total lymphocytes (mean ± SD).
Lower line values, absolute count (/microliter, mean ± SD).

For Leu7 (CD57) cells, NK cells, and cytotoxic T lymphocytes (CTL), the percentages of CD57+CD8+ subset ($p < 0.05$) and CD56+CD3- NK cells ($p < 0.05$) were significantly increased with advancing age (Table 5). The proportion of CD3+CD56+ CTL[10] remained unchanged even if the age advanced. Furthermore, NK cell subsets were analysed by CD57 and CD56 (Leu19) using two-color flow cytometry. Interestingly the percentage of CD56+CD57- NK cells was decreased with aging, and in contrast CD56+CD57+ NK cells in the older ($p < 0.01$) and middle-aged groups ($p < 0.05$) were significantly increased compared with those in the younger group (Table 6).

Table 5. CD57+CD8+ cells, NK cells, and CTL in young, middle-aged, and aging subjects

Group	CD57+, CD8+	CD56+, CD3- NK	CD56+, CD3+ CTL
Young	10.4 ± 4.5	12.8 ± 7.7	3.3 ± 1.5
	192 ± 97	257 ± 247	62 ± 33
Middle-aged	14.8 ± 3.3	17.4 ± 5.2	3.5 ± 1.3
	305 ± 160	303 ± 112	60 ± 26
Aged	17.4 ± 9.0*	21.8 ± 13.3*	4.3 ± 3.1
	349 ± 255*	393 ± 229	93 ± 84

*$p < 0.05$; compared with the young group.
Upper line values, % of total lymphocytes (mean ± SD).
Lower line values, absolute count (/microliter, mean ± SD).

Table 6. NK cell subsets in young, middle-aged, and aging subjects

Group	CD56+, CD57- NK	CD56+, CD57+ NK	CD56-, CD57+ NK
Young	11.3 ± 6.2 232 ± 189	7.5 ± 3.5 140 ± 82	6.3 ± 4.1 119 ± 99
Middle-aged	8.5 ± 2.9 153 ± 70	11.5 ± 4.7* 205 ± 102	9.8 ± 2.8 179 ± 86
Aged	8.2 ± 2.4 155 ± 60	19.0 ± 10.6** 366 ± 208**	9.5 ± 5.3 197 ± 151

*$p < 0.05$, **$p < 0.01$; compared with the young group.
Upper line values, % of total lymphocytes (mean ± SD).
Lower line values, absolute count (/microliter, mean ± SD).

Discussion

This study investigated the influence of aging on the lymphocyte subsets using two-color immunofluorescence staining of whole blood and four parameters flow cytometry. The use of whole blood assay avoids the use of density gradient centrifugation which might potentially alter the ratios of lymphocyte subsets. Moreover, this method can use a lot of monoclonal antibodies simultaneously from a small amount of blood so that the whole blood assay is suitable for this type of study.

In humans, as aging proceeds, immune function begins to decline after peak in adolescence. This decline may partly account for various abnormalities occurring exclusively or increasingly in the elderly. With advancing age, in general, responses to foreign antigens decline,[11] whereas those to autoantigens are enhanced.[12]

T cells, as shown before,[13-15] were slightly decreased as aging proceeded, in association with a decreasing tendency in the number of CD8+ T cells. With regard to function of T cells, activated suppressor T cells stimulated with Con A has been reported to decrease in aging humans.[16] These results suggest a decrease of CD8+ T cells with advancing age not only in number but also in function.

Analysis of our data, unlike previous reports,[13] revealed no differences among the three groups in either the percentage or absolute numbers of B cells.[15] There are no changes in the production of immunoglobulin in the presence of PWM,[17] as compared with younger subjects. Moreover, the aged men had an increased proportion of CD4+Leu8- T cells,[15] which mediate the majority of helper functions involved in B cell differentiation into plaque forming cells.[5,7,8] Consequently, B cells are considered to be functionally activated in the elderly men. This view agrees with much evidence that the incidence of autoantibodies increases with aging.[12]

It has been reported that the number of CD25+ T cells as well as the number of CD71+ T cells was not affected by aging, but that IL2 synthesis and CD25 expression are defective in the elderly.[18] The present study shows the proportion of CD38 antigen positive activated T cells are significantly decreased in aging humans, but both HLA-DR+[15] and HLA-DQ+ activated T cells tended to increase. HLA-DR antigen is a late activation marker which is expressed on the surface of T cells which have been exposed to a chronic stimulation with antigens.[9] These data indicate that protection of the body depending on cellular immunity, as well as capacity of IL2 synthesis and CD25 expression, declines with allowing constant invasion of antigens in elderly men.

Elevation of circulating CD57+CD8+ cells has been associated with AIDS-related complex (ARC),[19] long-term surviving patients afer allogeneic bone marrow transplantation,[20] and primary Sjogren's syndrome.[21] Ligthart et al. reported that this increase also occurred in aged individuals.[22] Cytomegalovirus (CMV) carrier status of healthy individuals (53 of 112 individuals had IgG-class CMV antibodies) causes a persistent increase in the numbers of CD57+CD8+ cells,[23] and there are the slight but various influences of the other herpes virus carrier status (Epstein-Barr virus, herpes simplex virus, and varicella-zoster virus) on CD57 cell subsets.[24] CD57+CD8+ cells mediate lectin-dependent and anti- CD3 induced cytotoxicity, and could be in vivo-primed CTL.[25] In the present study, CD57+CD8+ cells increased with advancing age. This indicates that humans become more liable to viral infections as they become older, leading to an increase in number of CD57+CD8+ cells.

Changes in number and activity in NK cells, which have non-specific killer activity against a wide range of cancer cells, can be closely related to occurrence and aggravation of diseases in aging humans. In general, CD57 (Leu7) antibody has been used in studies on NK cells.[26] Because CD57 antigen may express in part of CD8+ T cells,[27] CD56 antibody specific to NK cells[10] was used in this study. The results showed that CD56+CD3-cells which were exclusively NK cells increased with advancing age. Some studies of NK function in aged people suggest an increase in NK function.[28,29] These findings could be related to the increased number of NK cells found in our study. However, in the older age group CD56+CD57- cells which are the most active NK cells[27] were decreased, whereas CD56+CD57+ cells and CD56-CD57+ cells were in-

creased, both of which are known not to have so much, or little, active NK function.[27] This suggests that a total number of NK cells increases in aging humans, but the subpopulation of the most active NK cells decreased, suggesting a decline of resistance against viruses or bacteria or carcinogens in the elderly.

Alterations among lymphocyte subsets associated with aging are multifarious. This study carried out by taking advantage of monoclonal antibodies with multiparameter flow cytometry succeeded in providing deep insights into decline in lymphocyte subpopulations in aged subjects. Such changes in lymphocyte subpopulations should be taken into consideration not only to investigate etiology of various diseases in elderly humans but also to treat older patients.

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