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Normal values of peripheral lymphocyte populations and T cell subsets at a fixed time of day: a flow cytometric analysis with monoclonal antibodies in 210 healthy adults

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SUMMARY

Using monoclonal antibodies and flow cytometry percentages and absolute number of lymphocyte populations and T cell subsets were enumerated in peripheral blood collected between 8.00 and 10.00 a.m. from 210 healthy adults. Although absolute numbers of total lymphocytes did not differ depending on age and sex, the numbers of T (Leu-4$^+$) and natural killer (Leu-7$^+$) cells as well as their percentages showed negative and positive correlations, respectively, with age. In Leu-7$^+$ cells, the percentage was male-dominant irrespective of age, and the absolute number was male-dominant only in older subjects. Absolute numbers of suppressor/cytotoxic T (Leu-2a$^+$) and helper/inducer T (Leu-3a$^+$) cells and percentages of Leu-2a$^+$ cells were negatively correlated with age in females. In males, only the percentage of Leu-2a$^+$ cells was age-dependent. Leu-3a/Leu-2a was positively correlated with age in females, and was female-dominant depending on age. These results indicate that peripheral lymphocyte populations and T cell subsets vary remarkably in healthy adults even at a fixed time of day.

Keywords lymphocyte populations T cell subsets healthy adults circadian rhythm

INTRODUCTION

Lymphocyte populations and T cell subsets in the peripheral blood have been reported with increasing frequency by experimenters using monoclonal antibodies. Their normal values, however, are still controversial (Nagal, Chrest & Adler, 1981; Abo, Cooper & Balch, 1982; Mascart-Lemone *et al.*, 1982; Gupta, 1984). These reports contain no description of the time of day at which blood samples were collected. Lymphocyte populations other than natural killer (Leu-7$^+$) cells have a marked circadian rhythm (Ritche *et al.*, 1983), and each T cell subpopulation also seems to have a different circadian rhythm (Miyawaki *et al.*, 1984). Thus, it is possible that percentages as well as absolute numbers of lymphocyte populations and T cell subsets differ depending on the time of collection, resulting in the inconsistency.

We have collected blood at a fixed time of day and determined percentages and absolute numbers of lymphocyte populations and T cell subsets in a large number of healthy subjects over a wide range of ages, using flow cytometric analysis with monoclonal antibodies. This report describes the values in relation to age and sex.

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MATERIALS AND METHODS

Subjects. Two hundred and ten healthy volunteers who had no history of noticeable disease and had not received any medication that affects immune reactions were studied. There were 116 males and 94 females aged 12 to 94 years. Between 8.00 and 10.00 a.m., 15 ml of their blood was drawn from the medial cubital vein with a plastic syringe, and 10 ml was placed in a plastic tube containing 10 U of heparin (Novo Industry, Copenhagen, Denmark) for analysis of lymphocyte populations and T cells subsets. The remaining blood was used for estimation of total and differential counts of peripheral white blood cells.

Monoclonal antibodies to lymphocyte populations and T cells subsets. Fluorescein-labelled anti-Leu-2a (suppressor/cytotoxic T cells), anti-Leu-3a (helper/inducer T cells), anti-Leu-4 (all T cells) and anti-Leu-7 (natural killer cells) were purchased from Becton-Dickinson (Mountain View, CA, USA). All antibodies were diluted to 1:20 with RPMI-1640 (GIBCO, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and used within 30 min.

Cell preparations and immunofluorescein staining. The collected heparinized blood was diluted with an equal volume of phosphate-buffered saline (PBS). Twenty millilitre of the diluted blood was overlaid on 15 ml of sodium metrizoate Ficoll solution (Lymphoprep; Nyegaard & Co., Oslo, Norway) and was centrifuged at 400 ***g*** for 40 min at room temperature. The cells at the interface were collected, washed three times by addition of the same volume of PBS and centrifuged at 300 ***g*** for 10 min at 4°C, followed by resuspension in RPMI-1640 containing 10% heat-inactivated FBS at a concentration of 1×10^6 cells/ml. To the cell pellets (obtained from 400 μl of the resuspended cells after centrifugation at 300 ***g*** for 10 min at 4°C) 50μl of each diluted monoclonal antibody was added, and the resulting mixture incubated on ice for 40 min. Cells were washed a further three times with PBS and resuspended in 1 ml of PBS for flow cytometric analysis. Negative controls consisted of cells suspended in RPMI-1640-10% PBS instead of the diluted monoclonal antibodies.

Flow cytometry. After exposure to labelled antibody, the cells were analysed on a FACS 420 (Becton-Dickinson, Mountain View, CA, USA). After setting forward light scatter gates on the lymphocyte cluster, 5×10^3 or 1×10^4 cells were analysed. The numbers of lymphocyte populations and T cells subsets were estimated from fluorescence-positive cells with background cells subtracted, and their proportions were expressed as percentages of the analysed cells. Their absolute numbers were calculated from total and differential counts of peripheral white blood cells, with the total cells analysed on FACS 420 regarded as lymphocytes.

Statistics. The significance of correlation coefficients was tested by correlation coefficient analysis, and the difference of the mean values, by Student's *t*-test.

RESULTS

Table 1 shows percentages and absolute numbers of lymphocyte populations and T cell subsets in the peripheral blood in three age groups in healthy subjects of both sexes: young (39 years or less), middle-aged (between 40 and 59) and old (over 60) groups.

Absolute number of total lymphocytes in the peripheral blood

Absolute numbers of total lymphocytes in the peripheral blood did not differ depending on age or sex.

Percentages of lymphocyte populations and T cell subsets

The percentage of Leu-4$^+$ cells was negatively correlated with age in both sexes, showing a significant decrease in the middle-aged and old groups over the young group. A similar age-related decline was also found in Leu-2a$^+$ cells in females, but in males a significant difference was found only between the young and middle-aged groups. In contrast, no age-related change was observed in Leu-3a$^+$ cells, resulting in a positive correlation between Leu-3a/Leu-2a and age in females and a

Table 1. Lymphocyte populations and T cell subsets in the peripheral blood of healthy adults

	Sex	CC	Age groups		
			Young	Middle-aged	Old
			Mean (±s.d.) percentage		
Leu-4	M	−0·457[b]	68·6±8·4 (50)	63·4±11·0[c] (36)	58·4±12·1[d] (28)
Leu-4	F	−0·443[b]	70·4±9·5 (47)	63·6±11·3[c] (23)	60·8±8·8[d] (23)
Leu-3a	M	ns	41·6±7·8[g] (50)	44·0±9·8 (36)	40·0±9·3 (29)
Leu-3a	F	ns	45·7±9·8 (48)	42·8±9·5 (23)	42·3±7·2 (21)
Leu-2a	M	ns	26·5±7·5 (50)	22·0±6·4[d] (28)	24·1±8·3[h] (29)
Leu-2a	F	−0·432[b]	23·8±6·7 (46)	19·2±4·0[d] (22)	18·6±5·0[d] (22)
Leu 3a/Leu 2a	M	ns	1·7±0·7[g] (50)	2·2±0·7[c] (28)	1·9±0·8[g] (25)
Leu 3a/Leu 2a	F	0·246[a]	2·1±0·7 (48)	2·4±0·7 (22)	2·3±0·7 (20)
Leu-7	M	0·693[b]	15·4±7·6[g] (35)	24·0±9·3[d,g] (32)	33·9±11·0[d,f,h] (21)
Leu-7	F	0·623[b]	11·5±4·9 (31)	18·0±7·6[d] (23)	22·9±8·3[d] (18)
			Mean (±s.d.) numbers/mm³		
Leu-4	M	−0·318[a]	1540±400 (27)	1410±520 (30)	1240±480[c] (21)
Leu-4	F	−0·327[b]	1680±550 (30)	1290±420[d] (22)	1320±370[c] (16)
Leu-3a	M	ns	950±280 (27)	990±290 (30)	930±350 (19)
Leu-3a	F	−0·279[a]	1090±390 (30)	880±270[c] (22)	890±230 (14)
Leu-2a	M	ns	640±220 (27)	520±270[g] (24)	540±220 (21)
Leu-2a	F	−0·478[b]	560±230 (30)	380±120[d] (21)	420±160 (13)
Leu-7	M	0·397[b]	380±190 (22)	510±270 (27)	740±340[d,c,h] (16)
Leu-7	F	0·395[b]	290±140 (26)	380±210 (22)	450±170[d] (16)
Total	M	ns	2270±520 (27)	2200±720 (31)	2170±560 (21)
LC	F	ns	2320±650 (30)	2060±670 (22)	2140±550 (16)

CC correlation coefficient between age and percentage or absolute number of lymphocyte populations and T cell subsets; LC lymphocytes; ns Not significant. Age groups are as follows: young, < 39 years; middle-aged, 40–59 years; and old, > 60 years. Figures in parentheses are numbers of subjects.

[a]$P<0·05$ and [b]$P<0·01$; [c]$P<0·05$ and [d]$P<0·01$ *v* young group; [e]$P<0·05$ and [f]$P<0·01$ *v* middle-aged group; [g]$P<0·05$ and [h]$P<0·01$ *v* females.

difference in the ratio between the young and middle-aged groups in males. A sex-related difference in these cells was found in the young group for Leu-3a$^+$ cells and in the old group for Leu-2a$^+$ cells. Leu-3a/Leu-2a for males was lower than that for females in the young and old groups.

The percentage of Leu-7$^+$ cells showed a good positive correlation with age, showing a significant difference among all age groups in both sexes except between the middle-aged and old groups in females. It was significantly higher in males than females in every age group.

Absolute numbers of lymphocyte populations and T cell subsets

The absolute number of Leu-4$^+$ cells declined in an age-related manner in a similar way to the percentage except for a difference between the young and middle-aged groups in males. The numbers of both Leu-2a$^+$ and Leu-3a$^+$ cells were negatively correlated with age, and a significant difference was found between the young and middle-aged groups in females. A sex-related difference in the number was observed in Leu-2a$^+$ cells for the middle-aged group.

In the case of Leu-7$^+$ cells, the number showed a positive correlation with age in both sexes; it was significantly higher in the old group than both the young and middle-aged groups in males and higher than the young group in females. A sex-related difference was present only in the old group.

DISCUSSION

Miyawaki *et al.* (1984) reported that percentages of peripheral OKT-3$^+$ (all T) and OKT-4$^+$ (helper/inducer T) cells have a marked circadian rhythm. According to Ritche *et al.* (1983), this rhythm is also found in the percentage of Leu-7$^+$ cells as well as in absolute numbers of Leu-4$^+$, Leu-3a$^+$ and Leu-2a$^+$ cells. These facts may indicate that both percentages and absolute numbers of peripheral lymphocyte populations and T cell subsets differ depending on the time of day at which blood samples are collected. Thus, we have determined the normal values at a fixed time of day.

Although age- and sex-related differences have been reported in the normal values enumerated using monoclonal antibodies, the results are conflicting and unsatisfactory. The same results as in the present study were partly observed in previous reports. Nagel *et al.* (1981) showed an age-dependent decline in the percentage of OKT-8$^+$ (suppressor/cytotoxic T) cells, but not in that of OKT-4$^+$ cells and Abo *et al.* (1982) found a positive correlation of the percentage of Leu-7$^+$ cells with age. In contrast, the results for the percentages of OKT-8$^+$ cells and OKT-4$^+$ cells in ageing reported by Mascart-Lemone *et al.* (1982) was the reverse of ours, while Gupta (1984) did not find any age-related difference. In these reports, the time of day of sample collection is not clear. Inconsistency may occur when blood samples are collected at different times of day, because the circadian rhythm by lymphocytes is remarkable (Ritche *et al.*, 1983; Miyawaki *et al.*, 1983).

Mononuclear cells isolated from the whole blood by Ficoll-Hypaque density gradient centrifugation contain more than 25% monocytes (Soman & Kaplow, 1980). In healthy subjects, however, these contaminating monocytes may be neglected, because small monocytes with a flow cytometer cannot exclude were reported to constitute only 2–3% (Nishikawa, Sakaguchi & Mikami, 1984). The normal values of lymphocyte populations and T cell subsets in the present study are reliable.

In conclusion, age- and sex-related difference of peripheral lymphocyte populations and T cell subsets are remarkable in healthy subjects, even when they are enumerated at a fixed time of day; therefore normal values matched in age, sex and time of sample collection should be used for the study of their changes in diseases.

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