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Detection of Lymphocyte Subsets by Monoclonal Antibodies in Aged and Young Humans

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ABSTRACT

With the use of Leu-series monoclonal antibodies, peripheral blood lymphocyte subsets in aged and young humans were determined.
1) In comparison with young individuals, Leu-1$^+$ cells and Leu-2a$^+$ cells were decreased, whereas Leu-7$^+$ cells and Leu-3a/Leu-2a were increased in aged individuals.
2) No sex difference could be observed in lymphocyte subsets.
3) PHA response of lymphocytes showed a negative correlation with Leu-2a$^+$ cells and a positive correlation with Leu-3a/Leu-2a in aged individuals.

As for surface markers of human lymphocytes, heretofore E receptor has been chiefly employed as T cell marker[15], while complement receptor[33] or surface immunoglobulin (sIg)[28] has been primarily employed as B cell marker. T cells are from the standpoint of function further subclassified into subsets. Moretta et al[23], have reported that IgG-Fc receptor bearing cells (Tγ cells) and IgM-Fc receptor bearing cells (Tμ cells) are present in peripheral T cells and that Tγ cells have a suppressor function, while Tμ cells have a helper function. On the other hand, Evans, Schlossman et al[7,8], in analyzing peripheral T cells with the use of antibodies (anti-TH$_1$ and anti-TH$_2$) produced by immunizing rabbits with human peripheral T cells, have reported that TH$_2^+$ T cells chiefly contain suppressor T cells (Ts) and cytotoxic T cells (Tc), while TH$_2^-$ T cells mainly contain helper T cells (Th) and suppressor inducer T cells (Ti). By using the autoantibody detected in the serum of juvenile rheumatoid arthritis (JRA) patients, they have reported that TH$_2^-$ JRA$^+$ T cells chiefly contain Ti, while TH$_2^-$ JRA$^-$ T cells mainly contain Th[36]. However, these anti-sera presented a number of problems with respect to specificity, antibody titer and/or reproducibility.

In 1975 Köhler and Milstein[17] by fusing mouse antibody producing cells with mouse myeloma cells successfully developed a method of producing hybridoma which secretes antigen specific monoclonal antibody (MoAb) to resolve at a stroke the problem points of the heretofore employed anti-sera. This hybridoma technology has also been applied to the analysis of surface marker of lymphocytes, and at present many MoAb have been produced with some already available on the market. The major ones are Leu-series produced by Becton-Dickinson and OK-series produced by Ortho Company. A description will be made of Leu-series MoAb.

Anti-Leu-1 is a MoAb secreted from hybridoma clone L17F12 established by Engleman et al[6]. Leu-1 is a pan T cell marker which appears in almost all T cells ranging from thymocytes to peripheral blood T cells and is homologous to OKT-1[29]. Leu-4[20] and OKT-3[30] are pan T cell markers which are similar to

Leu-1.

Anti-Leu-2a and Anti-Leu-3a are MoAb which are secreted from hybridoma clone SK1 and SK3 established by Evans et al[9]. Leu-2a is a marker of Ts and Tc (Ts/c) which appears in approximately 80% of the thymocytes and in about 30% of the peripheral T cells (chiefly TH_2^+ T cells) and is homologous to OKT-8[18]. Leu-3a is a marker of Th and Ti (Th/i) which appears in approximately 70—80% of the thymocytes and about 60% of the peripheral T cells (chiefly TH_2^- T cells) and is homologous to OKT-4[18].

Anti-Leu-7 is MoAb secreted from hybridoma clone HNK-1 established by Abo et al[1]. Leu-7 is a NK cell and K cell marker which selectively reacts with large granular lymphocytes (LGL) reported by Saksela et al[34] to be a population having a high NK cell activity.

Anti-Leu-10 is MoAb secreted from hybridoma clone SK10 established by Jackson et al. Leu-10 is a B cell marker which appears in all B cells and some monocytes of the peripheral blood.

Anti-HLA-DR is MoAb secreted from hybridoma clone L243 established by Lampson et al[19]. HLA-DR is a human class II major histocompatibility complex and is a human homologue of mouse Ia antigen. It appears in all B cells, some monocytes and activated T cells in the peripheral blood.

In the present study, with the use of the foregoing six types of Leu-series MoAb, peripheral blood lymphocyte subsets were determined and the effect of aging and sex were examined. Reference is also made to the relationship to lymphocyte response against phytohemagglutinin (PHA), which is considered to be one of the T cell functions.

SUBJECTS

Peripheral blood was drawn from 46 aged donors not having any disease composed of 30 males and 16 females with a mean age of 68.4 ± 8.2 years and from 32 young donors composed of 16 males and 16 females with a mean age of 22.6 ± 2.9 years.

METHODS

Preparation of peripheral blood lymphocytes (PBL)

PBL were separated from heparinized peripheral blood by Ficoll-Hypaque gradient separation[3].

Detection of lymphocyte subsets by MoAb (Fig. 1)

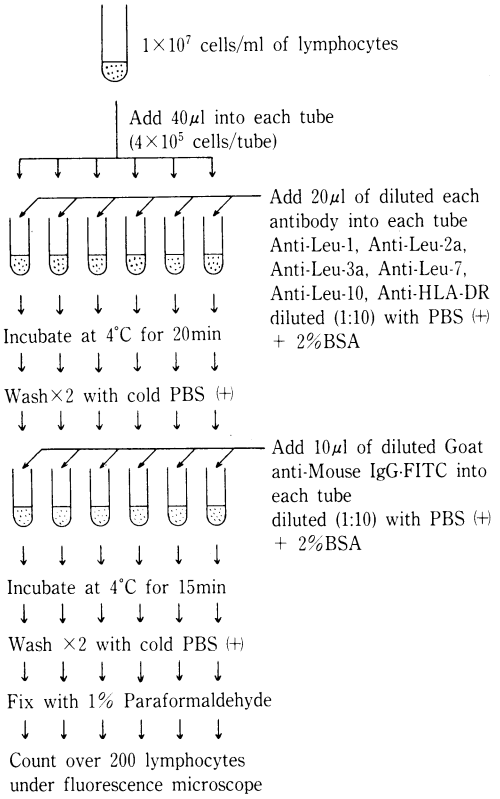

Fig. 1. Procedure of detection of lymphocyte subsets by monoclonal antibodies

A volume of 40 μl of PBL suspension of 1 × 10^7 cells/ml was each added to six microcentrifuge tubes (Labconic) and to each tube was added 20 μl of six types of Leu-series MoAb diluted tenfold by Ca^{++} Mg^{++} phosphate buffer saline [PBS(+)] containing 2% bovine serum albumin (BSA, Sigma), followed by incubation for 20 min at 4°C. After washing twice with cold PBS(+), 10 μl of affinity purified fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG antibody (Tago, Inc.) diluted tenfold by PBS(+) containing 2% BSA was added to each tube, followed by incubation for 15 min at 4°C. After washing twice with cold PBS(+), it was fixed with 1% paraformaldehyde and under the fluorescence microscope more than 200 lymphocytes were counted to determine the fluorescence positive rate. Moreover, the number of

peripheral blood leucocytes counted following Turk's stain was multiplied by the percentage of lymphocytes determined by May-Giemsa's stain to obtain the number of peripheral blood lymphocytes. This was multiplied by each fluoresence positive rate to obtain the absolute number of Leu^+ cells.

Detection of E-rosette forming cells

In accordance with the method of Kaplan et al[15], the percentage of cells which bind with sheep red blood cells (sRBC) treated with aminoethylisothiouronium bromide hydrobromide (AET) was determined under a microscope and these were assumed to be E-rosette forming cells.

Detection of Tγ cells

As described earlier, E-rosette forming cells were prepared and using Ficoll-Hypaque gradient separation method rosette forming cells were separated. sRBC were hemolyzed with 0.83% NH_4Cl solution and T cells were obtained. The percentage of T cells which bind with ox RBC sensitized with IgG fraction of rabbit anti-ox RBC (Japan Immunoresearch Laboratories Co.) was determined under the microscope and employed as Tγ cells.

Detection of sIg bearing cells

The percentage of cells which bind with FITC conjugated $F(ab')_2$ fragment goat anti-human Ig (Cappel Laboratories Co.) was determined under a fluorescence microscope and employed as sIg bearing cells.

Assay of NK cell activity

With the use of ^{51}Cr release assay method employing K-562 as target cells[4], incubation was made for 3 hr with effector: target ratio (E:T ratio) of 25:1 and % cytotoxicity was computed by the following equation:

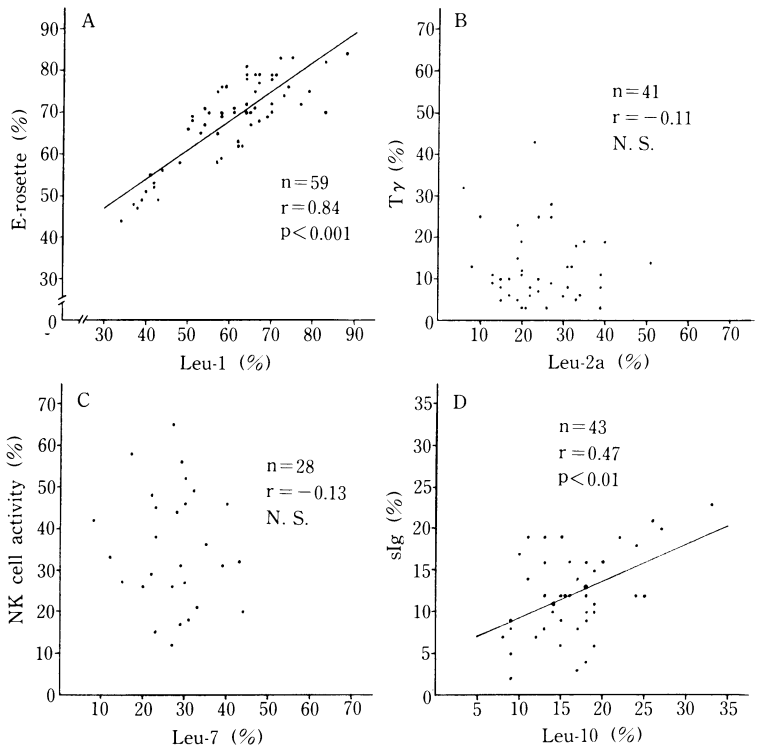

Fig. 2. Correlation between Leu^+ cells and other parameters. A: Leu-1 positive cells and E-rosette forming cells. B: Leu-2a positive cells and IgG-Fc-receptor bearing T cells (Tγ cells). C: Leu-7 positive cells and natural killer (NK) cell activities against K-562. D: Leu-10 positive cells and surface immunoglobulin (sIg) bearing cells.

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}$$

Assay of PHA response of lymphocytes

To PBL incubated for 96 hr with 1% PHA (Welcome) 0.4 μCi of ^{3}H-thymidine (Radiochemical Centre Amersham) was added and its incorporation was determined as count per minute (cpm).

RESULTS

Correlation between Leu$^+$ cells and other parameters (Fig. 2)

A significant correlation was observed between percentage of Leu-1$^+$ cells and percentage of E-rosette forming cells ($P<0.001$). No significant correlation could be demonstrated between percentage of Leu-2a$^+$ cells and percentage of Tγ cells nor between percentage of Leu-7$^+$ cells and NK cell activity. A significant correlation was observed between percentage of Leu-10$^+$ cells and percentage of sIg bearing cells ($p<0.01$).

Lymphocyte subsets reactive with MoAb in young and aged subjects (Table 1)

Table 1. Lymphocyte subsets reactive with monoclonal antibodies in young and aged subjects

	Young*	Aged*	P value
Percentage of Leu$^+$ cells (%)			
Leu-1	68.1 ± 6.8 **	60.6 ± 10.7	<0.001
Leu-2a	25.7 ± 6.2	21.2 ± 7.8	<0.01
Leu-3a	40.3 ± 6.2	39.9 ± 8.7	N.S.
Leu-7	16.9 ± 4.5	35.8 ± 9.2	<0.001
Leu-10	17.2 ± 4.7	17.8 ± 5.5	N.S.
HLA-DR	25.3 ± 7.0	26.4 ± 10.0	N.S.
Leu-3a/Leu-2a	1.66 ± 0.47	2.14 ± 0.84	<0.01
Absolute number of Leu$^+$ cells (/mm^3)			
Total lymphocytes	2741 ± 472	2277 ± 1025	<0.05
Leu-1	1866 ± 379	1358 ± 602	<0.001
Leu-2a	697 ± 179	482 ± 275	<0.001
Leu-3a	1107 ± 286	889 ± 398	<0.05
Leu-7	455 ± 125	885 ± 408	<0.001
Leu-10	467 ± 154	454 ± 256	N.S.
HLA-DR	689 ± 195	562 ± 235	<0.05

* 46 aged donors are compared to 32 young donors

** Results are expressed as mean ± 1 S.D.

The absolute number of PBL showed a significant decrease in aged donors. Leu-1$^+$ cells and Leu-2a$^+$ cells demonstrated a significant decrease in aged donors in both percentage and absolute number. Leu-3a$^+$ cells and HLA-DR$^+$ cells showed a significant decrease in aged donors only in absolute number. Leu-7$^+$ cells showed a significant increase in aged donors in both percentage and absolute number and Leu-3a/Leu-2a presented a significant increase in aged donors. Leu-10$^+$ cells did not present any significant difference.

Lymphocyte subsets reactive with MoAb in male and female subjects (Table 2)

In examining the subjects by sex, no significant difference could be demonstrated in absolute numbers of PBL and Leu$^+$ cells in both percentage and absolute number.

Correlation between Leu$^+$ cells and PHA response of lymphocytes in aged subjects (Fig. 3)

No significant correlation could be observed between PHA response of lymphocytes and the percentage of Leu-1$^+$ cells and Leu-3a$^+$ cells. PHA response of lymphocytes showed a signifi-

Table 2. Lymphocyte subsets reactive with monoclonal antibodies in male and female subjects

	Male*	Female*	P value
Percentage of Leu$^+$ cells (%)			
Leu-1	63.1 ± 10.2**	64.5 ± 9.7	N.S.
Leu-2a	22.6 ± 7.3	23.8 ± 7.8	N.S.
Leu-3a	39.6 ± 7.5	40.7 ± 8.1	N.S.
Leu-7	28.5 ± 12.5	25.8 ± 11.3	N.S.
Leu-10	17.4 ± 5.2	17.7 ± 5.1	N.S.
HLA-DR	25.9 ± 8.5	25.9 ± 9.5	N.S.
Leu-3a/Leu-2a	1.97 ± 0.77	1.90 ± 0.72	N.S.
Absolute number of Leu$^+$ cells (/mm^3)			
Total lymphocytes	2403 ± 932	2560 ± 772	N.S.
Leu-1	1500 ± 570	1662 ± 579	N.S.
Leu-2a	545 ± 253	607 ± 272	N.S.
Leu-3a	929 ± 344	1049 ± 398	N.S.
Leu-7	694 ± 396	682 ± 351	N.S.
Leu-10	449 ± 240	477 ± 170	N.S.
HLA-DR	600 ± 233	635 ± 219	N.S.

* 32 female donors are compared to 46 male donors

** Results are expressed as mean ± 1 S.D.

cant negative correlation with percentage of Leu-2a$^+$ cells and a significant positive correlation with Leu-3a/Leu-2a. However, this is a phenomenon only observed in aged donors and though not shown in the figure, this phenomenon could not be seen in young donors.

DISCUSSION

The correlation between Leu$^+$ cells and the heretofore employed parameters was examined. A correlation was observed between Leu-1$^+$ cells and E-rosette forming cells, but this is considered to be a natural result in view of the fact that both are T cell markers. There is no description in the literature on the correlation between Leu-1 and E-rosette, but it has been reported that there is a strong correlation between E-receptor and OKT-3 which is a pan T cell marker similar to Leu-1[5]. No correlation has been observed between Leu-2a$^+$ cells and Tγ cells, but it is considered at present that Tγ cells and Ts/c cells are completely different populations[31]. This also applies to Tμ cells and Th/i cells. No correlation can be observed between Leu-7$^+$ cells and NK cell activities, but this is because Leu-7$^+$ cells also contain other populations excluding NK cells and K cells[38] and this can also be explained by the probable difference in NK cell activity of individual lymphocytes. A correlation was observed between Leu-10$^+$ cells and sIg bearing cells. In general, the percentage of Leu-10$^+$ cells is higher than that of sIg bearing cells, but this is considered to be related to the fact that Leu-10$^+$ cells contain not only B cells but also some monocytes.

It has been reported that the absolute number of PBL decreases with aging[16,21], but some report that the number does not change[11,37]. According to the authors' data, a significant decrease was observed in aged donors. A number of reports have been made on the changes in lymphocyte subsets by aging. A study has been made by Nagel et al[24,25] using Leu-series and OK-series MoAb. According to their data, Leu-4$^+$ and OKT-3$^+$ cells which are pan T cells and Leu-2a$^+$ and OKT-8$^+$ cells which are Ts/c cells show a significant decrease by aging in both percentage and absolute number, while Leu-3a$^+$ and OKT-4$^+$ cells which are Th/i cells show a significant decrease by aging in only absolute number, but Leu-3a/Leu-2a and

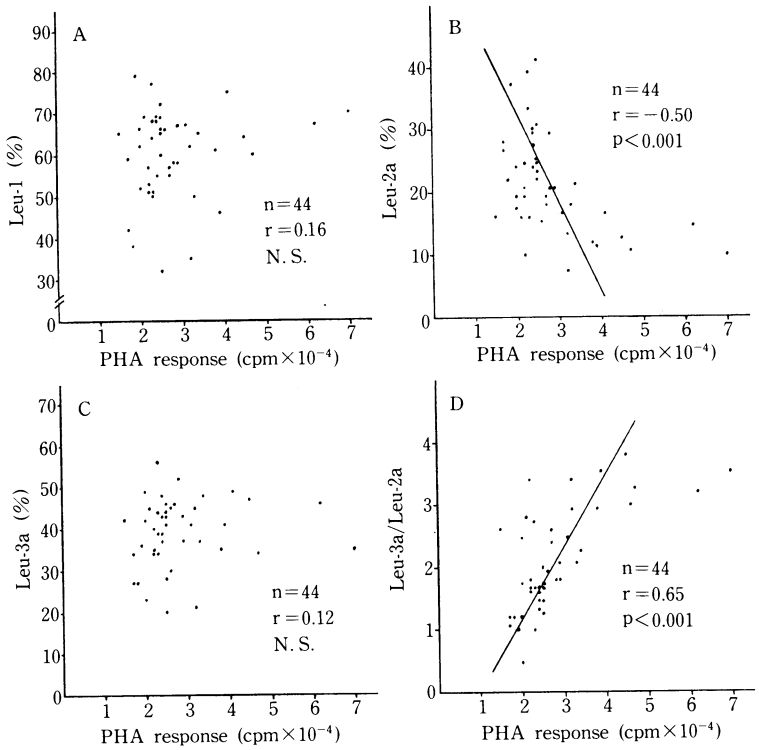

Fig. 3. Correlation between Leu$^+$ cells and PHA responses in aged subjects. A: Leu-1 positive cells B: Leu-2a positive cells C: Leu-3a positive cells D: Leu-3a/Leu-2a

and OKT-4/OKT-8 which are Th/i/Ts/c show a significant increase by aging. The results of their report are almost in agreement with the data presented by the present authors. However, Mascart-Lemone et al[22] have reported that the percentage of OKT-8$^+$ cells increases with aging, but Hallgren et al[12] have reported that OKT-3$^+$ and OKT-8$^+$ cells are not affected by aging. The relation between Leu-7$^+$ cells and aging has been previously reported by Abo et al[2]. According to their report, Leu-7$^+$ cells are not observed at time of birth, but gradually increase postnatally to reach a level of 10-20% at the age of 20—30 and a level of about 30% at the age of 60—70. These findings are almost consistent with data of the present authors, but the cause involved remains unknown. According to most of the reports on the relation between B cells and aging, B cells do not change in both percentage and absolute number[11,37]. These findings are in agreement with the data of the present authors. Furthermore, HLA-DR$^+$ cells decrease with aging only in absolute number, but this is considered attributable primarily to decrease in the absolute number of PBL.

Some reports have been made on the relation between lymphocyte subsets and sex. According to Nagel et al[25], no consistent trend can be observed in pan T cells, Ts/c cells, Th/i cells, and Th/i/Ts/c, but Mascart-Lemone et al[22] have reported that in aged females when compared to aged males the percentage of OKT-3$^+$ and OKT-4$^+$ cells is significantly increased, while the percentage of OKT-8$^+$ cells is significantly decreased. Hallgren et al[12] have reported to the similar effect. With regard to Leu-7$^+$ cells, Abo et al[2] have reported that on the average the male donors have shown a significantly elevated value when compared to the female donors. There have been no reports in the literature that a difference by sex has been observed in B cells and similarly no report has been made on the relation between HLA-DR$^+$ cells and sex. In contrast to the foregoing reports, accord-

ing to the data of the present authors no significant sex difference has been observed in all these lymphocyte subsets.

In aged donors, PHA response of lymphocytes showed a negative correlation to percentage of Leu-2a$^+$ cells and a positive correlation to Leu-3a/Leu-2a, but this phenomenon was not observed in young donors. Mascart-Lemone et al[22] have also made a similar report on the correlation between lymphocyte response to PHA, Con A and PWM and percentage of OKT-8$^+$ cells. This suggests that in aged donors the lymphocyte responses to mitogens are affected by Ts/c cells, in particular the effects of Ts cells, but it is unknown why this phenomenon is observed only is aged donors.

As for surface markers of human lymphocytes, many MoAb in addition to the aforementioned MoAb have been reported. As those specific to E-receptor, Leu-5[14], OKT-11[39], 9.6[13], etc have been reported, which are employed as a pan T cell marker. Leu-8[10], TQ1[32], etc are known to correspond to JRA and the possibility of separation of Th cells and Ti cells has been suggested. Furthermore, efforts are being made to separate Ts cells and Tc cells and such a possibility has been suggested for 9.3[13]. As NK cell markers, Leu-11a[27], NK-8[26], etc have been reported to have a specificity to LGL greater than Leu-7. Furthermore, as B cell markers, Leu-12, B-1[35], etc have been reported to have a higher specificity than Leu-10. We look forward in the future to the production of MoAb having a higher specificity and a higher accuracy by function.

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