Effects of Aging on the *In Vitro* Response of Human Lymphocytes to Interleukin-2

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Effects of aging on the *in vitro* responses of human peripheral blood lymphocytes (PBL) to interleukin-2 (IL-2, T-cell growth factor) were studied. Aged PBL showed a significantly lower response to phytohemagglutinin (PHA) than young PBL. On the other hand, proliferative responses to exogenous IL-2 showed by aged PBL were found to be in same extent as those of young PBL. In addition, cultured lymphoid cells propagated in IL-2 (CLC-IL-2) derived from both young and aged PBL showed a strong cytotoxic reactivity against the natural killer (NK) sensitive target K562.

Key Words: Interleukin-2 (IL-2), Aging, Natural killer (NK) activity, Mitogenic response

Changes associated with age in immune function in experimental animals and in humans have been described¹⁾. It is well established that lymphocytes from subjects over 65 years of age, incorporate less thymidine than those from young subjects when cultured with plant lectin²⁾. Recent studies revealed that mitogen-induced proliferation of murine and human T lymphocytes in culture is driven by IL-2, T cell growth factor, produced by T lymphocytes being stimulated with lectins or antigen. Some authors^{3, 4)} suggested that the proliferation of lymphocytes may depend upon their capacities to produce IL-2 and their abilities to respond to IL-2.

It was reported that *in vitro* incubation of human lymphocytes with IL-2 can induce the proliferation to their CLC-IL-2 and CLC-IL-2 thus derived were reported to exhibit a strong cytotoxic reactivity against the tumor cells resistant to lysis of natural killer cells and also against NK-sensitive tumor cells^{3, 5-7)}.

Little is known for the proliferative ability of aged PBL in the presence of exogenous IL-2 and the cytotoxic reactivities of CLC-IL-2 derived

from aged PBL. In the present study, PBL from the young and aged subjects were found to be able to propagate to a large number with IL-2 and CLC-IL-2 thus derived from aged PBL exhibit a strong cytotoxic reactivity against the NK-sensitive target cell K562 like young PBL, were reported.

MATERIALS AND METHODS

Subjects

Blood was obtained from healthy persons between 21 and 31 years in our laboratory and from those volunteers of ages ranging from 71 to 90 years old. Care was taken to choose the aged persons who were found to be healthy or apparently healthy without those diseases enough to affect their immune system.

Media

PRMI-1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) (RPMI/HI-FBS) was used unless otherwise noted.

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Click's medium was prepared as described⁸⁾.

Preparation of PBL

PBL were separated by means of Ficoll-Isoopaque density-gradient centrifugation⁹⁾. Adherent mononuclear cells were removed by incubation in 55 cm² tissue culture dishes (Falcon 3003, Div. Becton, Dickinson and Co., Cockeysville, MD.) in a 5% CO₂ atmosphere for 1 hr.

Preparation of human IL-2

Partially purified human IL-2 was kindly provided by Dr. Yoshikatsu Hirai, Institute of Otuska Pharmaceutical Co., Tokushima, Japan. It was obtained by culturing human spleen cells with PHA as previously described¹⁰⁾. Briefly, after 48 hr incubation, PHA-stimulated cells obtained were removed by centrifugation and the supernatants thus obtained were passed through 0.45 μ m filters to remove cellular debris. The supernatants were then precipitated by sequential ammonium sulfate precipitation, followed by DEAE column chromatography and by gel filtration on an Ultrogel AcA 54 column. Then the active portion obtained was exposed to pH 2.0 to destroy "immunological" interferon (IFN) activity. High concentrations of the purified IL-2 activities were found to be not directly mitogenic to normal mononuclear cells, thus further presence of lectin as judged no longer to occur. Furthermore, significant IFN activity was undetectable in these preparations. Units of IL-2 were determined as the reciprocal of the dilution degree that induce 50% of the maximal ³H-thymidine incorporation¹¹⁾ by the standard IL-2-dependent human cytotoxic T cell line. T cell line had been induced by mixed lymphocyte culture reaction between normal human peripheral T cells and mitomycin C-treated CESS cells and maintained in IL-2 for more than 16 weeks¹²⁾.

CLC-IL-2

PBL at 5×10^5 cells/ml were cultured with 10 μ g/ml PHA and 5 U/ml of IL-2 in Click's medium in tissue culture dishes (Falcon 3001). After 2 days of culture, cells were washed, counted in a hemocytometer and subcultured every 3 days at 1×10^5 cells/ml in Click's medium containing 5 U/ml of IL-2. In this study, we used 5 U/ml of IL-2, at which final concentration

was found to be optimal for the proliferation for PBL in the preliminary study using the concentrations of 0.2 to 15 U/ml of IL-2 for culture (data not shown). On the ninth and fifteenth day of culture, the cells at 1×10^5 cells/well were added to Microtest III culture plates (Falcon 3072) and incubated in the presence of 5 U/ml of IL-2 for 24 hr. The cells thus cultured were pulsed with ³H-thymidine (0.5 μ Ci/well; New England Nuclear, Boston, MA.) during final 6 hr of culture and then harvested. In other experiments on the twelfth day of culture, the cells were washed, resuspended in RPMI/Hi-FBS, and their cytotoxic reactivities were assessed against target K562.

Assay for cytotoxic activity

K562 cells, a myeloid cell line¹³⁾, were labeled with Na₂⁵¹CrO₄ (Daiichi Radio Isotope Labs., Ltd., Tokyo) as previously described¹⁴⁾ and used as targets. Briefly, cells were combined in a total volume of 1 ml of RPMI/Hi-FBS in plastic tubes (Falcon 2058). The tubes were centrifuged at 80 $\times$ G for 5 min and then incubated for 4 hr. After incubation, the tubes were recentrifuged at 800 $\times$ G for 5 min and 0.5 ml portion of the supernatants was removed and assayed for the percentage of released radioactivity. The percent of specific lysis was calculated as follows.

$$\% \text{ specific lysis} = \frac{\text{Experimental cpm} - \text{Control cpm}}{\text{Total cpm}} \times 100$$

Where experimental cpm is the mean value released in the presence of effector cells, control's is that released from target cells incubated with the medium alone, and the total cpm is the total amount of ⁵¹Cr incorporated into target cells. The value for control's released by target alone ranged from 5 to 10%.

PHA response of PBL

Two-tenths milliliters of PBL (5×10^5 cells/ml in RPMI/Hi-FBS) in each well of a microtest culture plate (Falcon 3072) was cultured in the presence of 10 μ g/ml PHA for 72 hr. The cells were pulsed with ³H-thymidine (0.5 μ Ci/well) during final 6 hr of culture and then harvested. Statistical analysis

Data (mean ± S.D.) were compared for statistical significance by means of Student's *t*-test and considered as significant if *p* < 0.05.

RESULTS

PHA response of PBL from young and aged donors

Thymidine incorporation by PBL from eleven young and twelve aged subjects incubated with PHA for 72 hr was measured. As had been previously found, thymidine incorporation was significantly less by aged PBL when compared with that of young PBL (Table 1).

Donor	Age	³ H-Thymidine Incorporation (cpm)
Young Adult (n = 11)	27 ± 3	106138 ± 30023
Aged (n = 12)	78 ± 5	51355 ± 21848

Table 1. PHA response of PBL from young and aged donors

Donors (Age)	Number of Cells (1 x 10 ⁵ /ml)				Cumulative Cell Number (3 - 15)
	Days of Culture				
	3 - 6	6 - 9	9 - 12	12 - 15	
Young Adult					
1 (21)	5.3	2.0	2.5	2.5	66.3
2 (23)	9.3	1.5	3.3	1.8	82.9
3 (24)	7.2	6.2	3.8	2.2	373.2
4 (25)	3.0	8.2	9.7	2.2	525.0
5 (25)	6.6	6.7	4.0	3.9	689.8
6 (26)	2.8	4.2	3.5	1.4	57.6
7 (27)	3.0	1.9	1.9	1.3	14.1
8 (28)	12.0	2.2	4.4	1.8	209.1
9 (31)	4.8	7.8	5.7	3.8	811.0
10 (31)	5.5	4.3	2.1	2.5	124.2
11 (31)	3.0	3.7	1.3	1.2	17.3
Mean (27)	5.7	4.4	3.8	2.2	270.0
Aged					
1 (71)	8.2	4.1	3.1	1.1	114.6
2 (72)	6.7	4.2	3.2	2.5	225.1
3 (73)	3.6	3.1	3.0	2.7	90.4
4 (73)	6.7	8.0	4.6	2.2	542.4
5 (75)	4.0	9.0	1.4	2.4	121.0
6 (77)	3.8	7.0	2.5	3.4	226.1
7 (78)	6.6	4.1	5.9	3.6	574.8
8 (79)	2.1	2.0	2.3	1.3	12.6
9 (80)	5.3	4.5	2.8	0.9	60.1
10 (81)	6.0	6.3	1.9	3.7	265.7
11 (83)	3.0	2.7	3.3	2.0	53.5
12 (90)	4.0	2.5	1.1	1.5	16.5
Mean (78)	5.0	4.8	2.9	2.3	191.9

Table 2. Expansion of PBL from young and aged donors with IL-2

Expansion of PBL from young and aged donors with IL-2

The proliferative abilities of PBL from young and aged subjects in IL-2 were shown in Table 2. After incubation with PHA and IL-2 for 2 days, the cells were washed and subcultured every three days at 1 × 10⁵ cells/ml with a change of fresh Click's medium containing IL-2. After PHA stimulation, PBL of both young and aged subjects can respond to exogenous lectin-free IL-2 and propagate to a large number in the presence of IL-2. No significant difference was found for the growth capacity between aged and young CLC-IL-2 in each subculture. The growth capacity varied among the individual culture. About 82% of cultures originated with young PBL expanded to 58- to 811-fold (mean for all young subjects, 270 ± 273-fold increase), whereas 83% of the cultures from aged PBL showed similar growth capacity (mean for all aged subjects, 192 ± 182-fold increase) over a period of 3-15 days. Two out of eleven (18%) from young subjects and 2/12 of the cultures from aged subjects failed to expand to 20-fold. There was no direct correlation between the growth capacity of the individual cultures and their cytotoxic reactivity (see below). In other experiments, proliferative ability of PBL in IL-2 was evaluated by the method of thymidine incorporation (Table 3). On the ninth and fifteenth day of culture, the cells were pulsed with thymidine in the presence of IL-2. No significant difference in thymidine uptake in response to IL-2 was observed between young and aged CLC-IL-2 on the ninth day and on the fifteenth day of culture, respectively.

Cytotoxic reactivity of PBL and CLC-IL-2 of young and aged donors

Cytotoxic reactivity was assessed against the

Donor	Age	³ H-Thymidine Incorporation (cpm)	
		Day 9	Day 15
Young Adult (n = 12)	28 ± 3	70432 ± 29390	57334 ± 26149
Aged (n = 14)	76 ± 5	66830 ± 36413	48589 ± 27617

Table 3. ³H-Thymidine incorporation by CLC-IL-2 from young or aged donors in response to IL-2

NK-sensitive target cell K562. The cytotoxic reactivity of fresh PBL in the aged group was $30.5 \pm 7.7\%$, which did not differ from that in the young group ($29 \pm 9.8\%$). CLC-IL-2 derived from the aged and the young groups showed strong cytotoxic reactivity (75.9 ± 3.5 and $77.3 \pm 3.2\%$, respectively). No significant difference was found between both groups (Table 4).

Donor	Age	Cytotoxicity (%)	
		PBL	CLC-IL-2
Young Adult (n = 11)	27 ± 3	29.8 ± 9.8	77.3 ± 3.2
Aged (n = 12)	78 ± 5	30.5 ± 7.7	75.9 ± 3.5

Table 4. Cytotoxic activity of PBL and CLC-IL-2 from young or aged donors against K562 targets

DISCUSSION

In the present study, we demonstrated that PBL from aged subjects were able to propagate to a large number with supplementation of IL-2. A decrease in lymphocyte responses of the aged humans and animals to T cell mitogens had been reported by several investigators^{1, 2, 15, 16}. The decreased response of PBL from aged subjects to PHA were observed in the present study and also in our previous study¹⁴. Gillis *et al.*¹⁵ pointed out that the impaired lymphocyte response to mitogens by aged human is due to defects in both the production and recognition of IL-2. They demonstrated that PBL from aged subjects showed either no change or even a decrease of thymidine incorporation by culture in the presence of PHA and IL-2 when compared with the case of culture in the presence of PHA alone. However, they did not investigate the number of propagated cells due to supplementation of IL-2 after PHA stimulation. Thoman *et al.*¹⁷ reported that spleen cells from old mouse produce less IL-2 in response to mitogenic stimuli but respond to exogenous IL-2. The results we obtained demonstrated that, after PHA stimulation, PBL from aged humans can respond to exogenous IL-2 as those from the young and proliferate to a large number by culture. Thymidine incorporations on the ninth

and fifteenth day respectively, showed no difference of proliferative abilities exerted by IL-2, between the young and aged subjects. Therefore, we considered that following interaction of PBL from aged subject with PHA stimulation, a receptor for IL-2 in PBL was expressed and PBL was induced to proliferate to CLC-IL-2 in the presence of exogenous IL-2 as those PBL from young subjects. There is no significant difference being found as their property among IL-2 receptor expressing cells between the young and aged subjects. On the other hand, it is worth noting that a small portion (18%) of the eleven young PBL cultures showed a relatively poor growth in the presence of IL-2. The apparent difference between mitogen response and proliferative ability with IL-2 is required to be performed.

Only few on age-associated change of the lymphocyte-mediated cytotoxic reactivity in humans is known. Our previous report¹⁴ and those of others^{18, 19} demonstrated that aged healthy subjects have similar normal cytotoxic reactivity against the NK-sensitive target cell K562 when compared with young controls.

The ability of human CLC-IL-2 to lyse the fresh autologous and allogeneic tumor cells *in vitro* has been reported by some authors^{5, 6}. Kedar *et al.*⁵ revealed that CLC-IL-2 are in high cytotoxic to K562 targets and also to various tumor cells without prior specific antigen stimulation. In the present study, we demonstrated that CLC-IL-2 exhibited a strong cytotoxic reactivity against K562 targets. In addition to this finding, CLC-IL-2 derived from aged PBL also showed similar potent cytotoxic reactivity against K562 as young controls.

The properties of the present NK-like cytotoxic cells grown under this culturing condition have not been fully defined. Kedar *et al.*⁵ reported that the cytotoxicity shown by CLC-IL-2 is attributable to propagation of NK cells. Sugamura *et al.*⁷ also revealed that human NK cells could be proliferated in the presence of IL-2, and cloned T cell lines thus obtained are able to possess NK activity. On the basis of our findings, NK-like activity of the present CLC-IL-2 was significantly diminished by treating with anti-leu 7 (monoclonal antibody to human NK cell antigens) and complements

(unpublished data). Human NK cells have been shown to express certain T cell antigens²⁰. Therefore, we considered that after PHA stimulation, human endogenous NK cells or their precursor cells could proliferate *in vitro* in the presence of IL-2. However, it is still not elucidated that whether the present NK-like cells being able to grow in IL-2 are identical to those NK cells occurring endogenously in peripheral blood, or not.

Judging from the findings obtained by the present authors, the immunity of aged subjects may be recovered by exogenous IL-2 and that is according to the reactivity to exogenous IL-2 being possessed by PBL from aged subjects as those PBL from young subjects.

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