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NATURAL KILLER (NK) ACTIVITY OF NORMAL HUMAN PERIPHERAL BLOOD LYMPHOCYTES AGAINST ERYTHROLEUKEMIC CELL LINE K 562*)

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ABSTRACT

Human peripheral blood lymphocytes of healthy donors showed significant levels of natural killer (NK) activity against erythroleukemic cell line K 562 in 3-hour ^{51}Cr release assays. The levels of NK activity showed much individual variation, which was considered to be an individual trait that varied little under physiological conditions. The NK activity of mononuclear leukocytes from cord blood was very low, but increased early in infancy to the adult level.

The effect of contamination of the lymphocyte preparation with granulocytes or red blood cells was examined. When red blood cells were added in a ratio of 10 : 1 or more, NK activity was significantly suppressed. On the other hand, contaminating granulocytes had no effect on NK activity. Pre-treatment of lymphocytes with Tris-buffered ammonium chloride drastically obliterated the NK activity of these cells; so this treatment should be avoided.

When monocytes were removed from mononuclear cell preparations, the NK activity increased in many cases, but the magnitude of augmentation varied from donor to donor and from day to day. The possibility of monocyte-NK cell interaction was discussed.

In order to minimize the day-to-day fluctuation of NK assay conditions, peripheral blood mononuclear leukocytes were cryopreserved. These cryopreserved cells retained more than 80% of their NK activity.

INTRODUCTION

It has long been known that normal lymphocytes, in the absence of disease, sensitization, or deliberate immunization of the lymphocyte donor, can destroy a variety of target cells cultured *in vitro*. This natural cytotoxicity has been treated as an artifactual background noise, which should be subtracted from the specific, disease-related cell-mediated cytotoxicity. Because it has been taken for granted by most immunologists that each immunologic phenomenon must be elicited by a particular, known immunogen, this natural cytotoxicity of normal unsensitized lymphocytes has been regarded as a nonspecific, nonimmunologic, artifactual phe-

*) 田中義人：正常ヒト末梢血リンパ球の K562 に対するナチュラル・キラー (NK) 活性について

nomenon.

However, this natural cell-mediated cytotoxicity has recently been extensively documented in humans[1-5] and rodents[6-12], and now this natural killer (NK) activity, or spontaneous lymphocyte-mediated cytotoxicity (SLMC) is thought to play a major role in the maintenance of homeostasis; i. e., resistance to tumor cells[13-15], allogenic bone marrow grafts[16], viral diseases[17,18], and regulation of differentiation of normal hematopoietic cells[19].

This paper describes the NK activities of peripheral blood lymphocytes from healthy human adults and children. A 3-hour ^{51}Cr release assay, in which the K 562 erythroleukemic cell line was used, was evaluated for its reproducibility.

MATERIALS AND METHODS

Preparation of effector cells:

Mononuclear leukocytes were isolated from the heparinized peripheral blood of healthy human donors by centrifugation with Ficoll-diatrizoate sodium gradient at 400×G for 30 minutes at room temperature. Cells were washed 3 times in phosphate-buffered saline (PBS) and suspended in Eagle's minimal essential medium (MEM No. 1, Nissui) supplemented with 10% fetal calf serum (FCS, Gibco). When red blood cells caused gross contamination, they were lysed by hypotonic shock with 0.2% NaCl for 15–20 seconds at room temperature. In some experiments, contaminating red blood cells were lysed by 0.83% Tris-buffered ammonium chloride treatment for 2–5 minutes at 37°C. These cells contained 80–85% lymphocytes and 15–20% monocytes with less than 2% granulocytes, as judged by peroxidase staining and morphology.

In order to remove monocytes, $6–8\times10^6$ mononuclear cells, suspended in 3 m*l* MEM with 10% FCS, were incubated at 37°C for 2 hours in 65 mm×15 mm Falcon plastic dishes. Nonadherent cells were harvested and were more than 96% lymphocytes. They were resuspended in MEM with 10% FCS. The viability of the effector cells, assessed by the trypan blue exclusion method, was always more than 95%.

Cell line:

The erythroleukemic cell line K 562, established from blast cells in the pleural effusion of a patient with blastic crisis of chronic myelogenous leukemia[20-23], was kindly provided by Dr. N. Kamada of the Department of Internal Medicine, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Hiroshima, Japan. The K 562 cells were grown in suspension culture in MEM with 10% FCS. Some K-562 cells were cryopreserved in anticipation of trouble in the continuously cultured and passaged strain.

Cryopreservation:

Mononuclear leukocytes isolated as described above were suspended in MEM with 25% FCS and put on ice. The cell suspensions were made up at twice the desired final concentration. Subsequent manipulations were carried out on ice. The 20% dimethyl sulfoxide (DMSO) solution was made up by adding 2.0 m*l* DMSO to 8.0 m*l* MEM with 25% FCS. An equal volume of the 20% DMSO solution was added to the cell suspension in a rapid, dropwise fashion, with mixing, over 30 seconds. The final cell concentration was $2–5\times10^7$/m*l* in 10% DMSO and 22.5% FCS. The cells were then dispensed in 1.0 m*l* aliquots into Nunc screw cap vials and immediately transferred to a programmed freezer (Planar Model R202/101R, G. B. Planar Ltd, Middlesex, England). The freezer was precooled to 4°C, and the cell suspensions were then cooled at a rate of −1°C/minute to −40°C and subsequently cooled to −90°C at a rate of −5°C/minute. The vials were then immediately transferred to a liquid nitrogen freezer for storage in vapor phase at −160°C.

Thawing and dilution of cryopreserved cells:

Cryopreserved vials were removed from the liquid nitrogen freezer and thawed rapidly in a 37°C water bath without shaking. The vials were kept at room temperature, and the dilution procedure to remove DMSO was initiated immediately. Subsequent steps were carried out at room temperature. The cell suspensions were diluted with a slow stepwise dilution procedure utilizing MEM with 25% FCS as the diluent. The diluent, one-twentieth of the original sample volume, was added dropwise with mixing. After one minute, the doubling volume was added. Subsequently, at one-minute intervals, a doubling volume of diluent was added 3 times. Then, after 5 minutes, enough diluent was added to achieve a final

dilution of 1 : 10. The cells were washed twice in diluents at 200×G for 10 minutes. They were resuspended in MEM with 10% FCS, and cell concentrations were adjusted for the cytotoxicity assay. The viability of the thawed cells, assessed by the trypan blue exclusion method, was always more than 90%.

The K 562 cells were cryopreserved and thawed and diluted as described above. The thawed and diluted K 562 cells were resuspended in MEM with 10% FCS and cultured for 3–4 days, passaged once, and subsequently used as the targets of cytotoxicity assay.

Cytotoxicity Assay:

Approximately 2×10^6 target cells (K 562) were labeled with 100 μCi of ^{51}Cr ($Na_2\ ^{51}CrO_4$, 1 mCi/m*l*, specific activity 250–450 μCi/mg Cr, New England Nuclear, Corp, Boston, Mass.) in 0.2 m*l* MEM with 10% FCS for 50 minutes at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The labeled K 562 cells were washed 3 times with cold PBS and resuspended in MEM with 10% FCS, and adjusted to 10^5 viable cells/m*l*.

The labeled cells were dispensed into wells of Nunc 96-well U-bottomed microtiter plates, each well receiving 0.1 m*l* containing 10^4 target cells. Then, equal volumes of various dilutions of suspensions of effector cells were added to triplicate wells, yielding ratios of effector to target cells (E/T ratio) of 6.25, 12.5, 25, 50, and 100. Control wells received 0.1 m*l* of medium for the determination of spontaneous ^{51}Cr release, or 0.1 m*l* of 0.5% Triton X-100 for the determination of maximum ^{51}Cr release.

The plates were incubated at 37°C in a humidified CO_2 incubator for 3 hours. Assays were terminated by centrifuging plates at 600 ×G for 5 minutes at room temperature, and 0.1 m*l* of supernatant was collected for counting in a well type gamma counter (Shimazu Model RAW-600, Shimazu, Japan).

Calculation of NK activity:

The NK activity (per cent specific lysis) was calculated from the following formula:

$$\text{per cent specific lysis} = \frac{\text{CPM (experimental)} - \text{CPM (spontaneous)}}{\text{CPM (maximum)} - \text{CPM (spontaneous)}} \times 100$$

where spontaneous and maximum release was determined as described above. Spontaneous release was usually less than 20% of the maximum release.

RESULTS

Representative assay done on a given day:

A representative 3-hour ^{51}Cr release cytotoxicity assay done on a given day is shown in Fig. 1. The effector cells were freshly isolated peripheral blood mononuclear cells. The NK activity (% specific lysis) increased linearly with the E/T ratio. This linear dose-dependency was seen until the E/T ratio was 200 : 1, but when the ratio was increased to 400 : 1 or more, the per cent specific lysis was the same as, or slightly lower than, that of the 200 : 1 ratio (data not shown, but confirmed from 9 separate experiments).

The individual variation of cytotoxic activities was significantly wide, e.g., at the E/T ratio of 50 : 1, the per cent specific lysis of 8 healthy

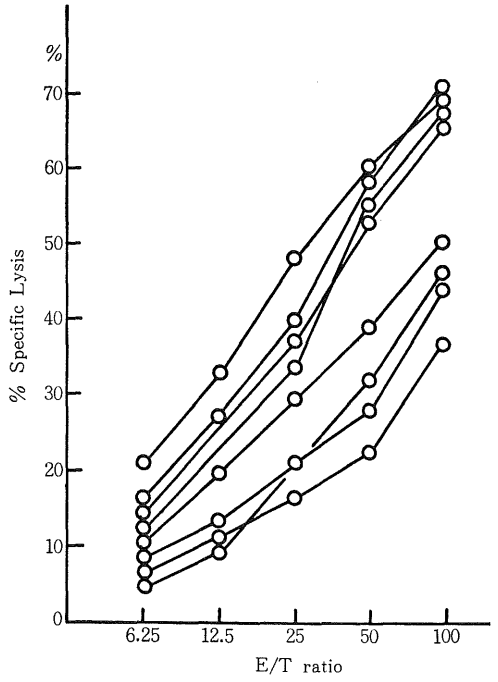

Fig. 1. Representative assay done on a given day. Mononuclear cells from 8 healthy adult donors were prepared by centrifugation on Ficoll-diatrizoate sodium gradient and allowed to react with ^{51}Cr-labeled K 562 cells for 3 hours at E/T ratios of 6.25 : 1, 12.5 : 1, 25 : 1, 50 : 1, and 100 : 1. Maximum release was 1355.4±14.3 cpm, and spontaneous release was 175.1±10.7 cpm (mean±S.D.) (n =6).

adult donors was 60.8, 57.7, 55.2, 53.1, 38.0, 31.8, 27.8, and 22.4%. However, individuals with high or low reactivity tended to maintain the same level of cytotoxic activity, and their order did not change in several assays done on different day (Fig. 9, see below).

Kinetic study of NK activity of peripheral blood lymphocytes:

To study the kinetics of NK cytotoxicity against K 562 cells, peripheral blood lymphocytes from 4 healthy adult donors were tested for various incubation times. As shown in Fig. 2, significant lysis was already detected after 15 minutes of incubation, and the per cent specific lysis increased as incubation continued. When the cells were allowed to react for 17 hours, the spontaneous release was 26.0 % of the maximum release, which was higher than after 4 hours or less of incubation (less than 15.0%), and was not optimal. The per cent specific lysis of the 3-hour assay was as high as that of the 4-hour assay.

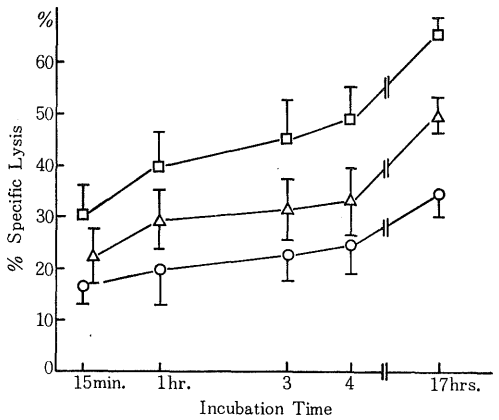

Fig. 2. Kinetic study of NK activity
Peripheral blood lymphocytes from 4 healthy adult donors were prepared by centrifugation on Ficoll-diatrizoate sodium gradient and monocytes were removed by adhesion on plastic dishes. They were incubated with ^{51}Cr-labeled K 562 cells at E/T ratios of 25 : 1 (□), 12.5 : 1 (△), and 6.25 : 1 (○). The curves show mean % specific lysis± S.D.

Effects of granulocyte and red blood cell contamination on the NK assay system:

In the present method of preparing effector cells, contamination with granulocytes was always less than 2%, and red blood cell contamination was usually negligible, but when the effector cells were obtained from some anemic patients, there was gross red blood cell contamination. Therefore, the effect of granulocyte and red blood cell contamination on the assay system was examined.

As shown in Fig. 3, granulocytes per se had no cytotoxic activity against K 562 cells, and contamination with granulocytes had no effect on the NK activity of the lymphocytes. However, gross contamination with red blood cells significantly suppressed NK activity. As shown in Fig. 4, there was no effect when the ratio of red blood cells to lymphocytes was 5 : 1, but when the ratio increased to 10 : 1 or more, significant inhibition was seen (about 70% inhibition).

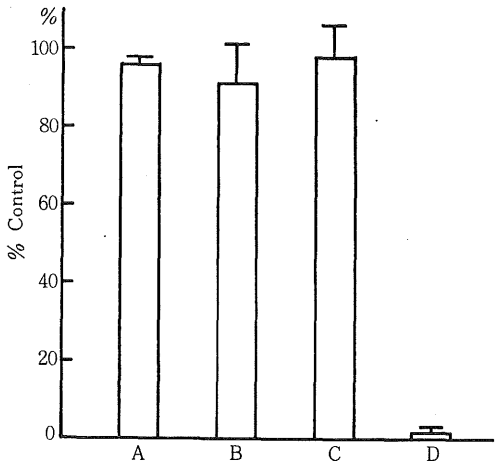

Fig. 3. Effect of granulocyte contamination
Peripheral blood lymphocytes from 3 healthy adult donors were prepared by centrifugation on Ficoll-diatrizoate sodium gradient, and monocytes were removed by adhesion on plastic dishes. Autologous granulocytes, prepared by the dextran sedimentation method, were added to each lymphocyte preparation, with ratios of granulocytes to lymphocytes of 0.25 : 1 (A), 0.5 : 1 (B), and 1 : 1 (C). The lymphocyte concentration was fixed at 5×10^5 /0.2 m*l*, so the E/T ratio was 50 : 1. Peripheral blood granulocytes (D) had no cytotoxic activity against K 562 cells at a granulocyte to target cell ratio of 50 : 1 (n=4).

Effect of pre-treatment of lymphocytes with ammonium chloride and hypotonic NaCl solution:

Because of the inhibitory effect of red blood

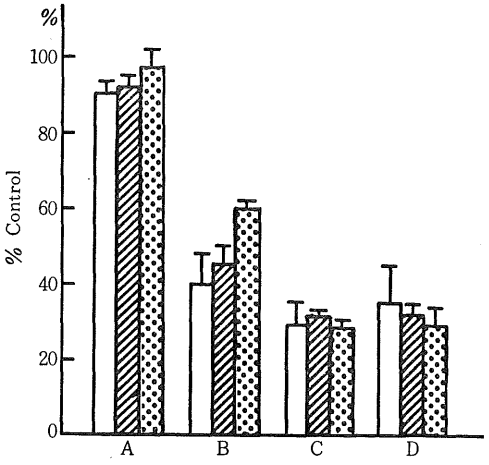

Fig. 4. Effect of red blood cell contamination
Autologous red blood cells were added to mononuclear leukocytes from 3 healthy adult donors, at ratios of red blood cells to mononuclear leukocytes of 5 : 1 (A), 10 : 1 (B), 50 : 1 (C), and 100 : 1 (D). They were incubated with ^{51}Cr-labeled K 562 cells for 3 hours at E/T ratios of 50 : 1 (□), 25 : 1 (▨), and 12.5 : 1 (▩).

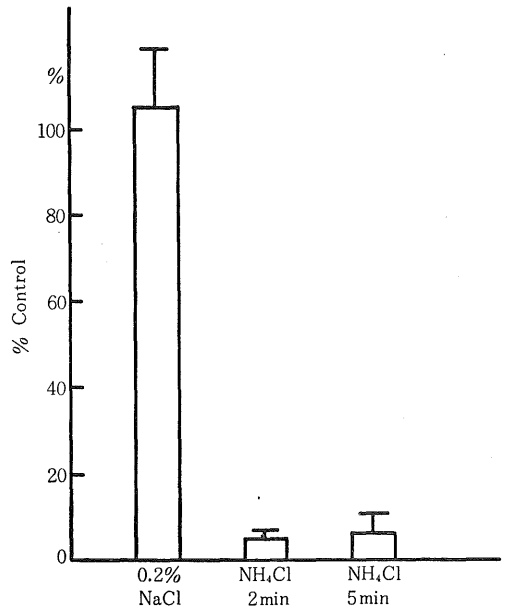

Fig. 5. Effect of ammonium chloride treatment
Peripheral blood mononuclear leukocytes from 5 healthy adult donors were treated with 0.83% Tris-buffered NH_4Cl for 2 and 5 minutes at 37°C. Hypotonic shock was done with 0.2% NaCl solution for 15–20 seconds at room temperature. These pretreated cells were then incubated with ^{51}Cr-labeled K 562 cells at an E/T ratio of 50 : 1 for 3 hours. The viability of post-treated cells was always more than 90% by the trypan blue exclusion method. The data show the mean % control ±S.D. (n=5).

cells on NK activity, they must be lysed, when there is gross contamination, before the cytotoxicity assay. Therefore, the effect of pre-treatment of the effector cells with 0.83% Tris-buffered NH_4Cl was examined. As shown in Fig. 5, pre-treatment of the mononuclear leukocytes with 0.83% Tris-buffered NH_4Cl for 5 minutes at 37°C drastically reduced NK activity (% specific lysis at E/T ratio of 50 : 1 was 1.7±2.4%; in controls it was 36.9±12.3%, $p<0.01$). When the duration of pre-treatment with ammonium chloride was shortened to 2 minutes, which was not sufficient to lyse contaminating red blood cells, a similarly significant reduction of NK activity was observed.

On the other hand, when the effector cells were pre-treated with 0.2% NaCl solution for 15–20 seconds at room temperature, there was no detrimental effect on NK activity.

Comparison of NK activity of monocy-deplet-

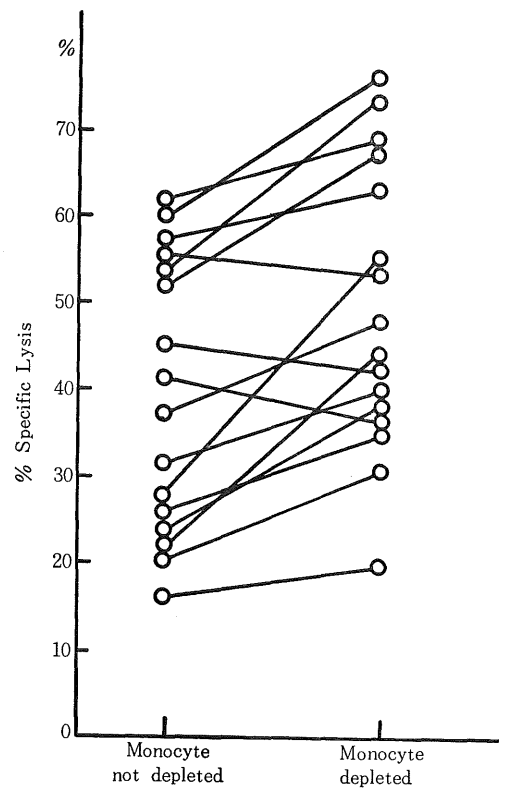

Fig. 6. Effect of monocyte depletion
Effector cells were prepared from 16 healthy adult donors as described in Materials and Methods, and monocyte-depleted and not-depleted cells were simultaneously incubated with ^{51}Cr-labeled K 562 cells at an E/T ratio of 50 : 1 for 3 hours.

ed and not-depleted peripheral blood mononuclear cells:

In 16 normal adult donors, the effect of the removal of monocytes on NK activity was studied. In 13 of the 16 cases, as shown in Fig. 6, the cytotoxic activity against K 562 cells increased after the removal of monocytes from the mononuclear population. The per cent increase in these 13 cases varied from 9.8% to 98.2%. In 3 of the 16 cases, on the other hand, some decrease in cytotoxic activity was observed. Moreover, the effect of the removal of monocytes in the same individual assessed on different days showed some day-to-day variation (Fig. 7).

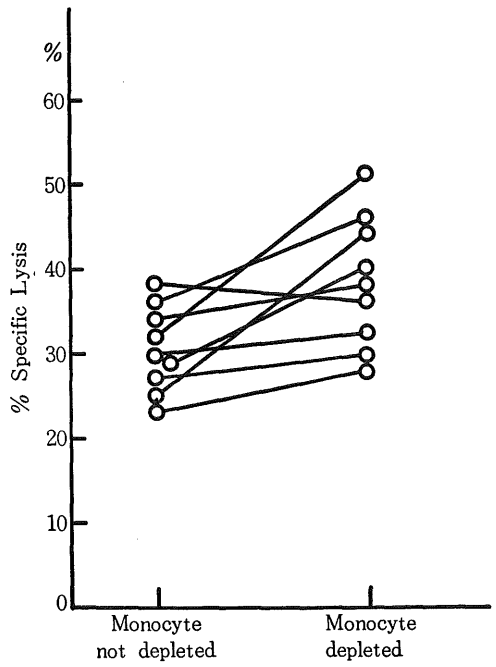

Fig. 7. Effect of monocyte depletion
The effector cells from one individual (Y. T.), prepared as in Fig. 6, were incubated with K562 cells serially on different days (E/T ratio=50 : 1) (n=9).

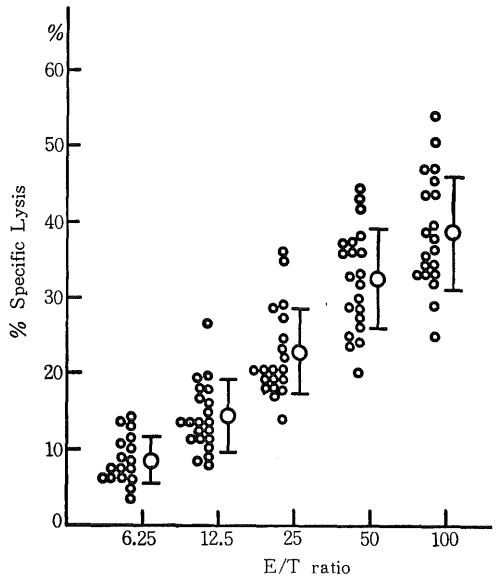

Fig. 8. Day-to-day fluctuation of NK activity
NK activity of the peripheral blood mononuclear cells from one healthy adult donor (Y. T.) was serially assayed on different dates for 6 months (n=21).

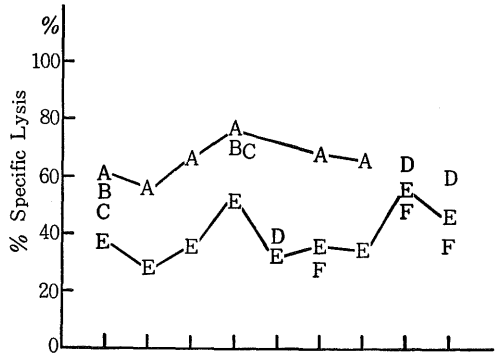

Fig. 9. Day-to-day fluctuation of the assay conditions
NK activity of peripheral blood mononuclear leukocytes from 6 healthy adult donors (A, B, C, D, E, and F) was assayed repeatedly on different dates (E/T ratio=50 : 1).

Individual day-to-day fluctuation of NK activity:

NK activity was serially assayed in one healthy adult donor over a 6-month period. As shown in Fig. 8, the level of NK activity fluctuated from day to day. When NK activity of the other normal adult controls was assayed repeatedly on different days, it was found to fluctuate similarly (Fig. 9).

Cryopreservation of effector cells:

In order to minimize the day-to-day fluctuation of the NK assay conditions, the effector cells were cryopreserved and stored for use as ideal controls.

The peripheral blood mononuclear cells from 5 healthy adult donors were cryopreserved and

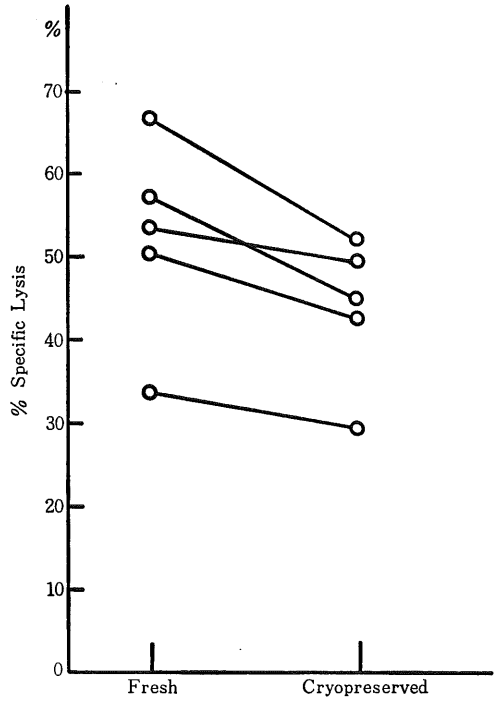

Fig. 10. NK activity of cryopreserved cells
NK activity of cryopreserved cells was compared with that of their fresh counterparts on the same day (E/T ratio=50 : 1) (n=5).

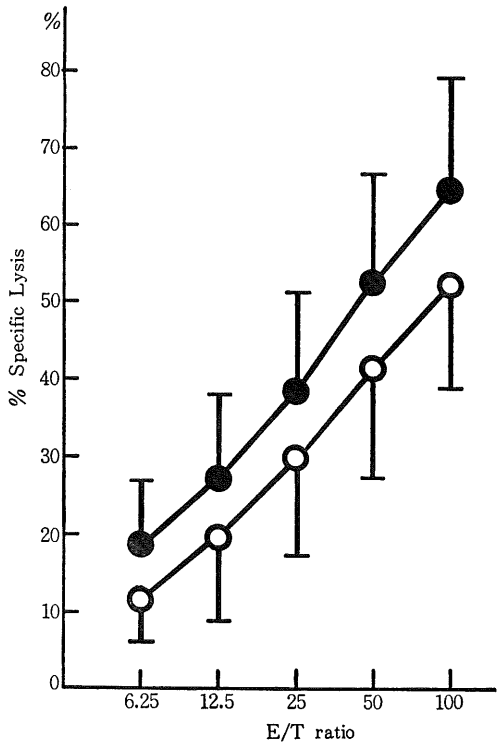

Fig. 11. NK activity of normal adult controls
The monocyte-depleted (●) and not-depleted (○) peripheral blood mononuclear leukocytes from 26 healthy adult donors were incubated with ^{51}Cr-labeled K 562 cells for 3 hours at various E/T ratios. The data show mean±S. D.

stored. Some of the cryopreserved cells from each donor were thawed within 30 minutes after cryopreservation, and their NK activities were compared with their fresh counterparts simultaneously prepared on the same day. As shown in Fig. 10, the NK activity against K 562 cells was well preserved after cryopreservation; the per cent specific lysis of the cryopreserved cells was 83.7±6.6% (mean±S. D.) of that of their fresh counterparts, and the order of activities of these 5 donors did not change after cryopreservation. After the cells had been cryopreserved and stored, their NK activities did not diminish further and were stable for at least 6 months (data not shown).

NK activity in normal healthy adult controls:

NK activity in 26 healthy individuals, 25-59 years old (mean age, 29.8±6.5 years), is depicted in Fig. 11 and Table.

When monocytes were removed, the NK activity increased slightly but not significantly ($p>0.05$). There was a wide range of NK activity from individual to individual. In some individuals, NK activities were assessed repeatedly on different days, and the order of these individuals with high or low reactivities did not change for at least 6 months, even though the actual per cent specific lysis caused by individual lymphocytes varied from day to day

Table NK activity of normal adult controls

E/T	NK activity* (mean±S. D.)	
	monocyte−depleted (n=18)	−not depleted (n=26)
100	64.7±14.5	52.3±13.5
50	52.7±14.4	41.3±14.2
25	38.2±12.8	29.8±12.8
12.5	27.2±10.5	19.4±10.9
6.25	18.3± 7.9	11.6± 5.9

* 3-hour ^{51}Cr relese assay (Target: K 562)

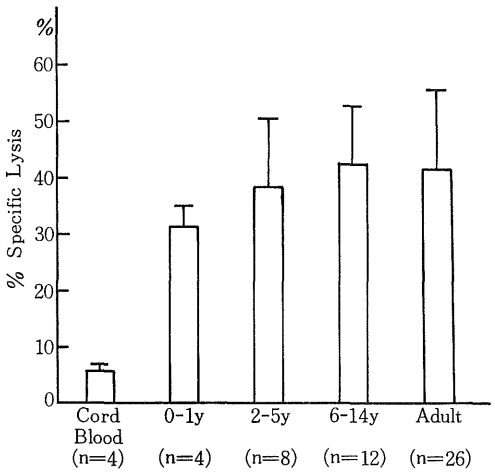

Fig. 12. NK activity of cord blood, infants, and children
The mononuclear leukocytes prepared from cord blood, infants, and children, were incubated with ^{51}Cr-labeled K 562 cells for 3 hours at E/T ratio of 50 : 1. The data show mean±S. D.

(Fig. 9).

NK activity in normal children, infants, and cord blood:

NK activity in various pediatric age groups is depicted in Fig. 12. It was significantly lower in mononucler cells from cord blood of mature healthy babies than in adult controls or other age groups ($p<0.01$). In infancy, NK activity was slightly lower, but this difference was not statistically significant ($p>0.05$). In children it was the same as in adult controls.

DISCUSSION

The data presented in this paper clarify many points relevant to the phenomenon of natural killer (NK) activity of human peripheral blood lymphocytes.

The K 562 cell line was chosen as the target because the susceptibility of this line to NK cytotoxicity is higher than that of any other line tested so far[4,24]. The line is very easy to maintain as a stationary suspension culture and has the advantage of being free of Epstein-Barr virus and HLA antigen[13,25].

Peripheral blood lymphocytes of normal human donors were found to have significant levels of NK activity against K 562 target cells. The levels of NK activity of different donors varied greatly (Figs. 1 and 10), as previously reported[26-28], and when NK activity of one individual was serially assayed on different days, the actual pre cent specific lysis fluctuated from day to day (Fig. 8). However, when the NK activity of many normal adults with high or low activities was tested repeatedly, the order of their activities did not change (Fig. 9). Therefore, in spite of certain day-to-day fluctuations in the assay conditions, the individual variability of NK activity of different donors is considered to be an individual trait that varies little under physiological conditions. Santoli et al.[29] reported that high and low NK activity was associated with certain HLA antigens. A broad population study of HLA hallotypes and cytotoxic activities may clarify this association.

A kinetic study of NK activity (Fig. 2) revealed significant lysis of the K 562 target already after only 15 minutes of incubation, and lytic activity increased with incubation time. Because the level of per cent specific lysis after 3 and 4 hours of incubation was almost similar, the 3-hour cytotoxicity assay was chosen for routine use, although the 4-hour ^{51}Cr release assay had been generally accepted as the standard method[2,5,30-34].

Granulocytes per se had no cytotoxic activity against the K 562 target in the 3-hour ^{51}Cr release assay, and contaminating granulocytes in autologous lymphocytes up to a ratio of 1 : 1 did not change the NK activity (Fig. 3). On the contrary, Santoli et al.[27] reported that the addition of granulocytes to purified lymphocytes resulted in a dose-dependent inhibitory effect on the NK activity of lymphocytes. They used the RDMC cell line, derived from a human rhabdomyosarcoma as their target, and this may be the reason for the discrepancy between our results and theirs.

The adverse effect of contamination with red blood cells on the NK assay system has not been previously reported. As shown in Fig. 4, when the contamination ratios were 10 : 1 or more, significant inhibition of NK activity was observed at every E/T ratio examined. This inhibitlon was thought to be mainly due to the crowding effect of the contaminating red blood cells, but some other mechanism could not be excluded, because 2.5×10^6 red blood

cells were not inhibitory at an E/T ratio of 50 : 1, while the same number of red blood cells did have an inhibitory effect at E/T ratios of 25 : 1 and 12.5 : 1.

The action of ammonium chloride in lysing red blood cells without apparent damage to non-erythroid cells is in common use by many immunologists to remove contaminating red blood cells from leukocyte preparations[35,36]. In the NK assay system, this treatment has been used routinely by many investigators, but no mention has been made of the detrimental effect of ammonium choride on NK effector cells[3,4,6,37-40].

As shown in Fig. 5, treatment of lymphocyte preparations with Tris-buffered ammonium chloride drastically reduced the NK activity of these cells. On the other hand, hypotonic lysis of red blood cells with 0.2% NaCl solution had no adverse effect on NK cytotoxicity, and this treatment was thought to be a suitable red blood cell lysing method for the NK assay system. Eremin et al.[41] reported a similar detrimental effect of $KHCO_3$-buffered NH_4Cl on the human NK assay system, in which the CLA-4 lymphoblastoid cell line was used as the target. Also in the murine NK assay system, Savary et al.[42] found that Tris-buffered NH_4Cl treatment significantly reduced the cytotoxic activity of mouse splenic NK cells and concluded that pretreatment of effector cells with ammonium chloride had only a detrimental effect on NK cell cytotoxicity and should therefore be avoided.

It is well known that there is a close relationship between the mononuclear phagocytic system and the lymphocyte system[43]. Macrophage-lymphocyte interaction is thought to be important in determining the fate of various immune reactions. In some situations, monocytes and/or macrophages can facilitate some lymphocyte-mediated immune responses, but in other situations, they can suppress the same function of lymphocytes.

As shown in Figs. 6 and 11, monocyte depletion of the mononuclear cell population resulted in the augmentation of NK activity in most but not all cases. The augmentation of human NK activity by monocyte depletion with carbonyl iron and magnetism and/or plastic surface adhesion has been reported by many investigators[3,27,30,40,44]. The degree of augmentation, however, varied from individual to individual, and a decrease in NK activity after monocyte depletion was observed in some cases (Fig. 6). When NK activities of many healthy donors were compared before and after monocyte depletion, the order of reactivity changed; i.e., some individuals with low activities changed to high reactors after monocyte depletion, and *vice versa*. Moreover, when the effect of monocyte depletion was assessed in the same individual on different days, the degree of augmentation varied from day to day (Fig. 7). It is not clear why the effect of monocyte depletion on NK activity differed from inividual to individual and from day to day. It seems unlikely that this augmentation was due simply to a relative increase in the number of NK cells following the removal of monocytes and some adherent lymphocytes by plastic surface adhesion. Some regulatory role of monocytes on NK activity has been suggested, and this regulatory activity of monocytes might differ from donor to donor in accordance with their *in vivo* situation. Although K 562 cells were reported not to be lysed by unstimulated human peripheral blood monocytes in the 4-hour ^{51}Cr release assay[45,46], there have been some indications that monocytes play an important role in human NK activity. In spite of many possible hypotheses of human monocyte-NK cell interaction, no evidence has been presented as to their precise mutual relationship *in vivo*. In the rodent system, on the other hand, this macrophage-NK cell relationship has been more clearly demonstrated. The administration of antimacrophage agents, e.g., silica particles or carrageenan, to mice or rats resulted in a decrease in splenic NK activity[12,16,19,47]. Murine macrophages activated by pyran copolymer or adriamycin suppressed NK activity in the spleen, although the same agents have also been shown to be able to augment NK activity[48-50]. Therefore, macrophages are thought to play an important role in regulating NK activity, sometimes augmenting, and sometimes suppressing it. The precise relationship between NK cell activity and macrophages, however, is not yet understood.

When comparing the results of ^{51}Cr release assays performed on different dates, it was difficult to determine whether the differences in reactivity reflected true differences of *in vivo* activity or were caused by altered assay con-

ditions. The quantitative comparison of NK activities in different donors was thought to be possible only when the assays were done on the same day. To overcome this problem, we attempted to cryopreserve peripheral blood mononuclear cells from different donors and from the same donor on different days. If cryopreserved cells can be stored with their functional reactivity intact, these cells can be used as ideal controls, and if many samples from normal donors or patients are cryopreserved, they can be assayed at any time.

As shown in Fig. 10, NK activity against K 562 targets in the 3-hour ^{51}Cr release assay was well maintained after cryopreservation. Other investigators have reported the detrimental effect of cryopreservation on NK activity against K 562 targets, in which different methods of cryopreservation were used[51,52]. They observed about 50% loss of NK activity after cryopreservation, but in this experiment NK activity after cryopreservation was stable for at least 6 months. Therefore, cryopreserved cells can be used safely as control effectors in the NK assay system.

NK activity against K 562 targets was significantly lower in mononuclear cells from cord blood, but rose early in infancy to the level in adult controls (Fig. 12). This early development of NK activity was in contrast to the findings in mice in which NK activity was reported to be totally absent at birth and then increased in young mice, the highest activity being in spleen cells from 5- to 8-week old mice[7,8]. Levin et al.[26] reported that NK activity of human cord blood against HBT-3 and ALAB targets was low or absent in the 3-hour ^{51}Cr release assay. Sato et al.[53] also reported low NK activity of cord blood lymphocytes, using monolayer targets such as RSb and RSa in a 16-hour ^{51}Cr release assay. Therefore, the low or absent NK activity of human cord blood is a general characteristic and not restricted to the K 562 target.

It is not known why NK activity in human cord blood lymphocytes is low or absent. Cantor et al.[54] reported that sera from newborn mice inhibited NK activity *in vitro*; this inhibitory activity was maximal at 4-7 days after birth. This blocking material had a molecular weight of approximately 70-80, 000 daltons. Örn et al.[55] reported that alpha-fetoprotein was a potent inhibitor of interferon-induced NK cell activation *in vitro*. Therefore, it seems a testable hypothesis that NK cell deficiency of cord blood lymphocytes is caused by endogenous alpha-fetoprotein, considering that the molecular weight of alpha-fetoprotein is 72, 000 daltons.

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