

## Natural Killer Activity of Normal Human Peripheral Blood Lymphocytes

### I. Inhibitory effect of ammonium chloride solution<sup>\*)</sup>

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### ABSTRACT

The inhibitory effect of ammonium chloride pretreatment of human peripheral blood lymphocytes on natural killer (NK) activity to myeloid leukemic cell line K-562 was studied. The more dilute the ammonium chloride solution, the less was the inhibitory effect on NK activity. This inhibitory effect was weaker when effector cells were treated at 4°C than at 37°C. Inhibited NK activity recovered partially after incubation for 24 hours.

### INTRODUCTION

Human peripheral blood lymphocytes from normal donors, without previous immunization, have cytotoxic activity against some *in vitro* tumor cell lines (eg, K-562). This "natural" killer activity of normal lymphocytes is believed to play a major role in host defence mechanism against virus infection and malignant diseases<sup>3, 4)</sup>. Previously, we reported that red blood cells contaminating effector cell preparation inhibited NK activity, so red blood cells had to be removed. However, when 0.75% Trisbuffered ammonium chloride solution was used to remove red blood cells, NK activity was greatly inhibited<sup>7)</sup>. The mechanism of this inhibitory effect of ammonium chloride is not known<sup>2, 6)</sup>. In this study, designed to clarify this point, normal peripheral blood mononuclear cells were pretreated with ammonium chloride solution under various conditions.

### 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 min-

utes 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). These cells were about 90% lymphocytes and 10% monocytes with less than 1% granulocytes, as determined by peroxidase staining and morphology.

#### *Target cells:*

Leukemic cell line K-562, established from blastic cells in the pleural effusion of a patient with blastic crisis of chronic myelogenous leukemia<sup>4)</sup>, was grown in suspension culture in Eagle's MEM with 10% FCS.

#### *Cytotoxicity assay:*

Approximately  $2 \times 10^6$  target cells (K-562) were labeled with 100  $\mu$ Ci of  $^{51}\text{Cr}$  ( $\text{Na}_2 \text{ } ^{51}\text{CrO}_4$ , 1 mCi/ml, specific activity 250-450  $\mu$ Ci/mg Cr, New England Nuclear, Corp, Boston, Mass.) in 0.2 ml MEM with 10% FCS for 50 minutes at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$ . The labeled K-562 cells were washed 3 times with cold PBS and resuspended in Eagle's MEM with 10% FCS, and adjusted to  $10^5$  viable cells/ml. The labeled cells were dispensed into wells of Nunc 96-well U-bottomed microtiter plates, each well receiving 0.1 ml containing  $10^4$  target cells. Then equal

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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, and 50. Control wells received 0.1 ml of medium for the determination of spontaneous  $^{51}\text{Cr}$  release, or 0.1 ml or 0.5% Triton-X 100 for the determination of maximum  $^{51}\text{Cr}$  release. The plates were incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  for 3 hours. Assays were terminated by centrifuged plates at  $600\times\text{G}$  for 5 minutes at room temperature, and 0.1 ml of supernatant was collected for counting in a Pakard Type 5110 gamma counter.

#### Calculation of NK activity:

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

per cent specific lysis

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

where spontaneous and maximum releases were determined as described above. Spontaneous release was usually less than 15% of maximum release.

#### Pretreatment of ammonium chloride solution:

A 0.75% isotonic ammonium chloride solution was prepared by adding 9 vol of 0.83% ammonium chloride (0.83g ammonium chloride in 1 liter double distilled water) to 1 vol of Tris buffer (20.594 g Tris base in 1 liter double distilled water), and the pH was adjusted with 1 N HCl to 7.4<sup>1</sup>. By replacing 4.5, 6.75, 7.875, and 9 vol of 0.91% NaCl, 0.37%, 0.19%, 0.09%, and 0% ammonium chloride solution were prepared respectively. And each 9 vol was added to 1 vol of Tris buffer, and the pH was adjusted with 1 N HCl to 7.4. As controls, Tris buffer (pH 7.4) and PBS (pH 7.4) were used.

#### Pretreatment of effector cells:

Monoclear leukocytes ( $5-10 \times 10^6$ ) were incubated with 3 ml of 0.75% Tris-buffered ammonium chloride at  $4^\circ\text{C}$ ,  $25^\circ\text{C}$ , and  $37^\circ\text{C}$  for various durations (a few second, 2, 5, and 10 minutes). After incubation, 7 ml of PBS was added and centrifuged at  $100\times\text{G}$  for 5 minutes and washed 2 times in PBS, and resuspended in Eagle's MEM supplemented with 10% FCS.

#### Incubation of ammonium chloride treated cells:

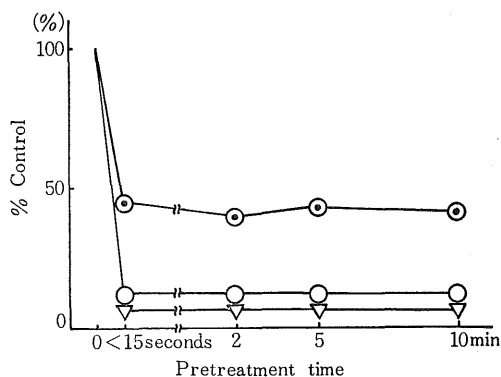
Monoclear leukocytes were treated with 3 ml of 0.75% ammonium chloride solution for 10

minutes at  $37^\circ\text{C}$ . After treatment, 7 ml of PBS was added and centrifuged at  $100\times\text{G}$  for 5 minutes. After washing 2 times in PBS, cells were resuspended in Eagle's MEM supplemented with 10% FCS, and incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  for 24 hours. After incubation, cells were centrifuged at  $100\times\text{G}$  for 5 minutes and resuspended in Eagle's MEM supplemented with 10% FCS and used as effector cells for NK activity assay. Simultaneously, freshly prepared mononuclear cells were incubated as described above and used as controls. The viability of incubated cells, both ammonium chloride-treated and -untreated, assessed by trypan blue exclusion method, was more than 96%.

## RESULTS AND DISCUSSION

When mononuclear leukocytes were pretreated with 0.75% Tris-buffered ammonium chloride solution at  $37^\circ\text{C}$  for 10 minutes, NK activity was almost completely inhibited. NK activity was not inhibited by the pretreatment with PBS, Tris buffer, or 0.91% NaCl.

The influence of temperature and duration of pretreatment were examined. As shown in Fig. 1, when the cells were pretreated at  $25^\circ\text{C}$  and  $37^\circ\text{C}$ , NK activity was completely inhibited. At  $4^\circ\text{C}$ , on the other hand, inhibition of NK activity was about 60%. The degree of NK

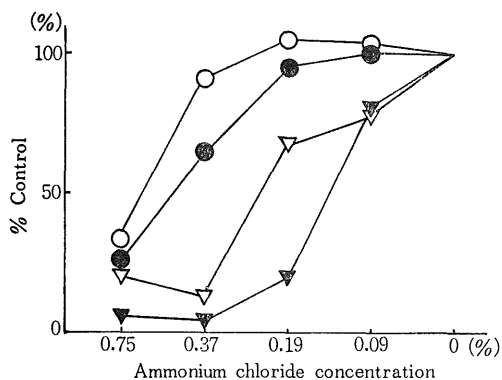


**Fig. 1.** Inhibitory effect of 0.75% ammonium chloride solution:

Pretreatment temperature were  $4^\circ\text{C}$  ( $\bullet-\bullet$ ),  $25^\circ\text{C}$  ( $\circ-\circ$ ), and  $37^\circ\text{C}$  ( $\nabla-\nabla$ ). E/T ratio was 50:1. The per cent control of NK activity was determined relative to NK activity without ammonium chloride pretreatment in simultaneous assays. These data are representative of similar experiments.

activity inhibition after a few seconds pretreatment was the same as after 10 minutes of pretreatment. Surprisingly, at 25°C and 37°C, NK activity was completely inhibited by only a few seconds of pretreatment.

The concentration of ammonium chloride solution was changed. As shown in Fig. 2, the more dilute the ammonium chloride solution, the less was the inhibitory effect. Mononuclear leukocytes were pretreated with ammonium chloride solution of various concentrations at 4°C and 25°C. At 4°C, a moderate inhibitory effect was expressed with 0.37% ammonium chloride solution, but almost no inhibitory effect was expressed with 0.19% or 0.09% ammonium chloride solution. At 25°C, the inhibitory effect of 0.37% ammonium chloride solution was the same as that of 0.75% ammonium chloride solution, but a moderate inhibitory effect was expressed with 0.19% and 0.09% ammonium chloride solutions. As shown in Fig. 1, the degree of inhibition with 0.75% ammonium chloride solution was clearly different between 4°C and 25°C. An obvious dif-



**Fig. 2.** Inhibitory effects of various concentration of ammonium chloride solution:

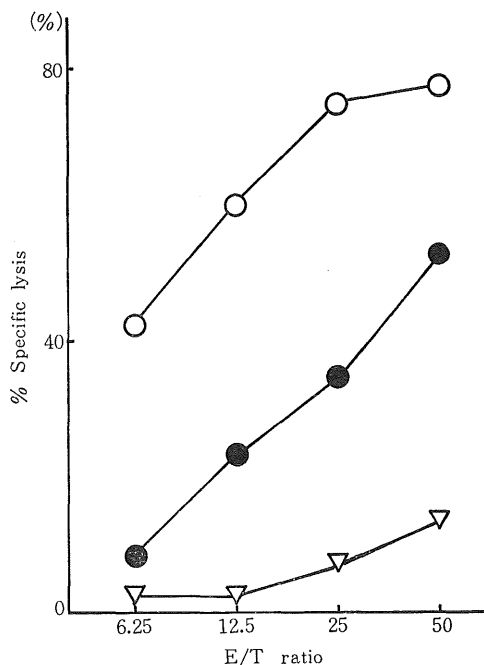
Various concentrations of ammonium chloride solution were prepared as described in materials and methods. Each ammonium chloride solution was Tris-buffered, isotonic, and at pH 7.4.

The temperatures and duration of pretreatment were as follows; 4°C, a few seconds (○—○); 4°C, 5 minutes (●—●); 25°C, a few seconds (△—△); and 25°C, 5 minutes (▼—▼).

Tris buffer, PBS, and Tris-buffered 0.91% NaCl had no inhibitory effect on NK activity. The per cent control of NK activity at each concentration was determined relative to NK activity at 0.91% Tris-buffered NaCl. These data are representative of similar experiments.

ference was also observed with various ammonium chloride solutions. Namely, at 4°C, no inhibitory effect was observed with 0.19% and 0.09% ammonium chloride solution pretreatment. On the other hand, at 25°C, moderate inhibition was observed. These experiments show clearly the inhibitory effect of ammonium chloride solution is closely related to the temperature and duration of pretreatment.

Recovery after incubation of NK activity inhibited by ammonium chloride solution is shown in Fig. 3. After mononuclear leukocytes were pretreated with 0.75% ammonium chloride solution at 37°C for 10 minutes, cells were incubated in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> for 24 hours. As a



**Fig. 3.** Recovery after incubation of NK activity inhibited by ammonium chloride pretreatment:

After mononuclear leukocytes were pretreated with 0.75% ammonium chloride solution at 37°C for 10 minutes, cells were incubated in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> for 24 hours (●—●). Control mononuclear leukocytes without pretreatment were incubated simultaneously (○—○). Pretreated mononuclear leukocytes without incubation were also prepared (△—△). NK activities of each mononuclear leukocyte are shown. The per cent specific lysis was calculated as described in materials and methods. Shown are data from 4 similar experiments.

control, mononuclear leukocytes without pretreatment were incubated simultaneously. Pretreated mononuclear leukocytes without incubation were also prepared. NK activity of pretreated mononuclear leukocytes without incubation was completely inhibited. The degree of NK activity of pretreated mononuclear leukocytes after incubation was almost 60% of NK activity of untreated mononuclear leukocytes after incubation. Thus, NK activity inhibited by ammonium chloride pretreatment was partially restored after incubation.

In summary, NK activity was markedly inhibited by pretreatment with ammonium chloride solution. The degree of inhibition varied with the temperature and duration of pretreatment. After incubation, inhibited NK activity was partially restored. At present time, the mechanism of inhibition is still not known, and further experiments are needed to explain it.

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