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Separation of Human Natural Killer Cells by Temperature Sensitivity and Soybean Agglutinin

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Introduction

It has been reported that natural killer (NK) cells are capable of destroying virus-infected cells and tumor cells without prior sensitization[8,9,11,12,16,17]. Great interest is shown in the role of NK cells as a host defense against viruses and tumors before the specific immune response appears[10,19]. In viral infections and in tumor-bearing patients, fever is a common symptom. However, it is still controversial whether fever is beneficial or harmful to patients, although whole body hyperthermia therapy has clinically been used to treat malignant neoplastic diseases[6,13]. Therefore, we attempted to examine the effect of various temperatures (37°C, 40.5°C and 43°C) on NK activity of human peripheral mononuclear cells (MNC). In the present study, we show that at least two NK subpopulations can be distinguished by temperature sensitivity; one subpopulation loses NK activity at 40.5°C, whereas the other is not affected. NK cells represent a heterogeneous family of lymphocytes, and have been separated into several subpopulations by their surface markers or by their properties such as adherence to nylon wool and ability of forming rosette with sheep erythrocytes[1,2,7,20,21].

Recently, a new approach for the separation of lymphocyte subpopulations by lectins has been established. Soybean agglutinin (SBA) has been used for the separation of B cells and T cells in mice[14]. In the present study, we show that SBA provides a useful tool for a separation of human NK subpopulations.

Materials and Methods

Preparation of effector cells: Human MNC were obtained from heparinized peripheral blood by Ficoll-Paque (Pharmacia, Sweden) gradient and suspended in RPMI 1640 medium plus 10% fetal calf serum (FCS).

Preparation of target cells: A human NK sensitive tumor cell line, K 562, was maintained as suspension culture and used in the [^{51}Cr] release assay as a target. Before usage, target cells were washed, and 1.5×10^6 cells were resuspended in 0.3 ml medium. Target cells were labeled

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with 150 μCi $Na_2[^{51}Cr]O_4$ (New England Nuclear, Boston, MA.) by means of incubation for 60 min at 37°C.

Cytotoxicity assay: Labeled target cells (1.3×10^4/100 μl) and effector cells (5×10^5/100 μl), which had been suspended in RPMI 1640 medium with 10% FCS, were then cocultured in 96 wells of "U" bottom microtiter plates (Corning, New York) for 4 hours at 37°C in 5% CO_2. After the incubation, the cell-free supernatants were collected using the Titertec Supernatant Collection System (Flow Lab, Irvine, Scotland) and counted by gamma counter. The spontaneous release was determined by adding medium to target cells, and it did not exceed 15%. Maximal release was determined by adding detergent to target cells. Specific lysis was calculated according to the following formula:

$$\% \text{ Specific lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

Standard error of the triplicates did not exceed 5%.

Separation of MNC by SBA: Separation of MNC by SBA (E.Y. Lab. CA USA) was performed by the modified method described by Y. REISNER et al.[15]. In brief, MNC suspension (6×10^7/0.2 ml) was incubated with SBA (2 mg/ml in 0.2 ml) for 5 min at room temperature. The cells were then layered on top of 8 ml of 50% FCS in RPMI 1640 medium. After 30 min at room temperature, the bottom (agglutinated, SBA^+) and top (unagglutinated, SBA^-) fractions were collected separately. The cells were then suspended in 0.2 M galactose in PBS. After 5 min at room temperature, the cells were collected by centrifugation, washed twice with galactose, and again twice with RPMI 1640 medium, and resuspended in RPMI 1640 medium plus 10% FCS at a concentration of 5×10^6/ml.

Results

Human MNC in RPMI 1640 medium with 10% FCS were incubated for various durations at various temperatures. After the incubation, cell viability was evaluated by a dye exclusion test using trypan blue. As shown in figure 1, heating below 43°C for 4 hours had no effect on the cell viability, whereas cell viability decreased after a 3 h-incubation at 45°C.

MNC (5×10^6/ml) in RPMI 1640 medium were incubated at 37°C, 40.5°C and 43°C for 0.5–4 hours. Then each NK activity was assayed. NK activity of MNC from a donor (S.I.), after incubation below 39°C, showed no change during a 4 h-incubation. At 43°C, however, it disappeared almost completely within 1 hour. At 40.5°C it was reduced to about half by 1 h-incubation, but NK activity exhibited no more reduction in spite of further incubation (Table 1 and Figure 2). To confirm that these observations were not restricted to the MNC from a special donor (S.I.), MNC from various healthy donors (22–36 years old) were examined. In all cases, NK activity at 40.5°C was reduced to about half to two-third of that at 37°C, and at 43°C it disappeared almost completely (Table 2).

The next step was to examine NK activity of MNC after long (20 hours) incubation. MNC were incubated at 37°C, 40.5°C and 43°C for 4 hours (first incubation). Then some of them were used for the assay of NK activity. The rest was incubated at 37°C in 5% CO_2 for 20 hours

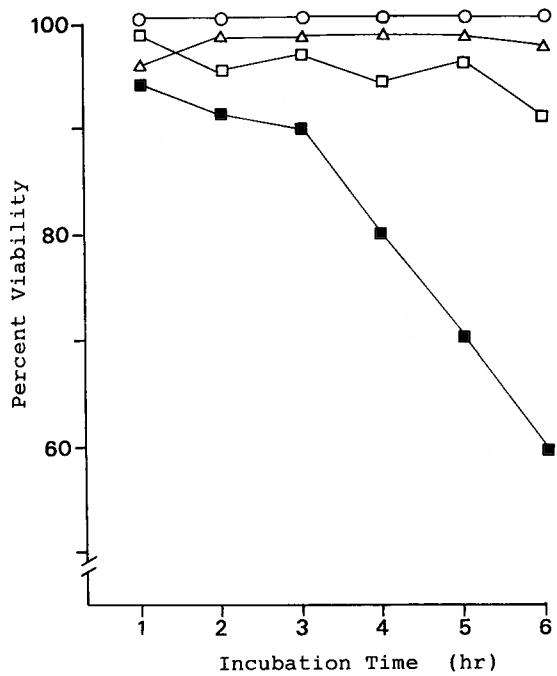

Fig. 1. Viability of MNC after incubation at 37°C (○), 40.5°C (△), 43°C (□) and 45°C (■). The cell viability was evaluated by a dye exclusion test using trypan blue.

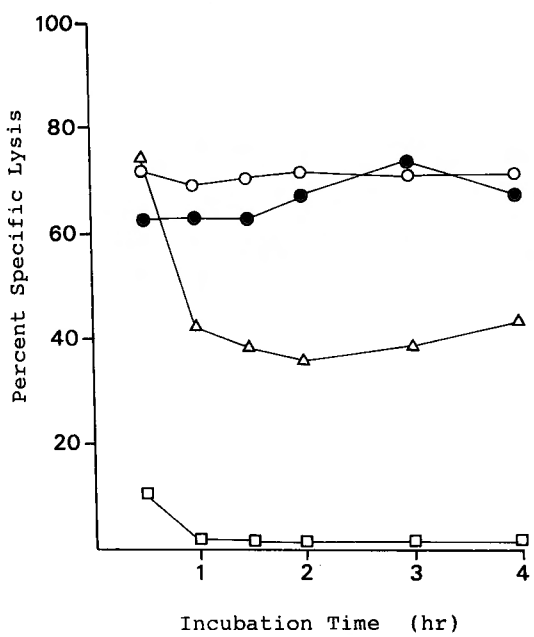

Fig. 2. NK activity of MNC after incubation at 37°C (○), 39°C (●), 40.5°C (△) and 43°C (□).

Table 1. NK activity of MNC* after incubation for various durations at various temperatures.

Experiment No.	Temperature (°C)	% Specific lysis of K 562						
		Incubation time (hr)						
		0.5	1	1.5	2	3	4	6
1	37						72.4	
	40.5	73.4	41.7	39.6	35.2	37.2	43.4	
	43	10.0	1.6	0	1.2			
2	37				60.2		58.6	
	40.5	61.5	37.6	20.8	26.2	36.9	40.3	
	43	2.3	2.9					
3	37							63.4
	40.5				27.1	28.9	34.2	34.7
	43				3.5		6.5	0.9
4	37		64.0		68.8			
	40.5		28.9	28.1	38.2			
	43		2.7	6.6	7.9			
5	37				63.1			
	40.5				33.3		22.8	
	43				0.5	1.1	4.3	

* MNC were obtained from a donor (S.I.).

(second incubation), and NK activity was assayed when the cell viability of MNC was more than 90%. Even in the control group (first incubation at 37°C), NK activity was reduced to about 50–80% by the second incubation only. MNC, first incubated at 40.5°C, maintained NK activity during the second incubation and came to have almost the same NK activity as the control group. NK activity of MNC, first incubated at 43°C, remained low or increased slightly (Table 3).

MNC were separated by SBA into agglutinated (SBA^+) and unagglutinated (SBA^-) fraction. SBA^+ fraction contained 55 to 88%, and SBA^- fraction was comprised of 12 to 45% of the total

Table 2. NK activity of MNC after 2 h-incubation.

Donors	% Specific lysis of K 562		
	Incubation temperature		
	37°C	40.5°C	43°C
H.T.	55.6	26.7	4.8
T.N.	47.3	16.8	4.1
J.S.	30.5	16.6	2.9
K.S.	44.6	30.1	5.0
T.K.	55.9	39.9	4.4
M.I.	56.8	42.9	6.0
Y.S.	26.9	17.5	5.7
N.N.	44.2	22.2	4.8
K.O.	58.9	40.2	1.6
R.J.	30.9	23.7	5.7

MNC from ten healthy donors were incubated for 2 hours at 37°C, 40.5°C and 43°C, then their NK activity was assayed.

Table 3. NK activity of MNC after first and second incubation.

Donors	Temperature of first incubation (°C)	% specific lysis of K 562	
		After first incubation	After second incubation
S.I.	37	63.1	31.4
	40.5	33.3	29.7
	43	1.1	7.2
S.I.	37	53.5	33.3
	40.5	40.9	33.3
	43	2.6	1.4
N.S.	37	44.6	30.1
	40.5	30.1	32.3
	43	5.0	5.8
T.K.	37	55.9	45.6
	40.5	39.9	37.7
	43	4.4	10.4

MNC were incubated at 37°C, 40.5°C and 43°C for 4 hours (first incubation). Then they were incubated further at 37°C in 5% CO_2 for 20 hours (second incubation).

Table 4. NK activity of MNC separated by SBA

Donors	% Specific lysis of K 562		
	Unseparated	SBA^-	SBA^+
S.I.	60.5	67.7	38.8
S.I.	52.3	54.3	44.4
J.S.	39.8	39.0	28.7
M.I.	44.2	60.1	38.0
Y.S.	16.2	19.1	12.9

MNC from four donors were separated by SBA. Then NK activity of unseparated, SBA^- and SBA^+ fractions was assayed.

number of MNC The SBA^- fraction showed stronger NK activity than the SBA^+ fraction (Table 4). The temperature sensitivity of SBA-separated fractions was studied. As shown in figure 3, after a 2 h-incubation at 40.5°C, NK activity of both fractions was reduced to approximately 50%, compared with the activity after the incubation at 37°C. After a 2 h-incubation at 43°C, they lost NK activity almost completely.

Discussion

In this study, heat sensitivity of NK cells was examined. Although death will occur in patients with fever of over 43°C, MNC were still alive at 43°C after a 4 h-incubation. However, MNC incubated at 43°C for only 1 hour lost their NK activity almost completely. Several groups have reported the effects of heat on human lymphocytes[4,18]. AGARWAL and GUPTA reported that DNA replication of T cells in response to phytohemagglutinin was enhanced at 40°C while the response of non-T cells to pokeweed mitogen was unaffected or decreased[3].

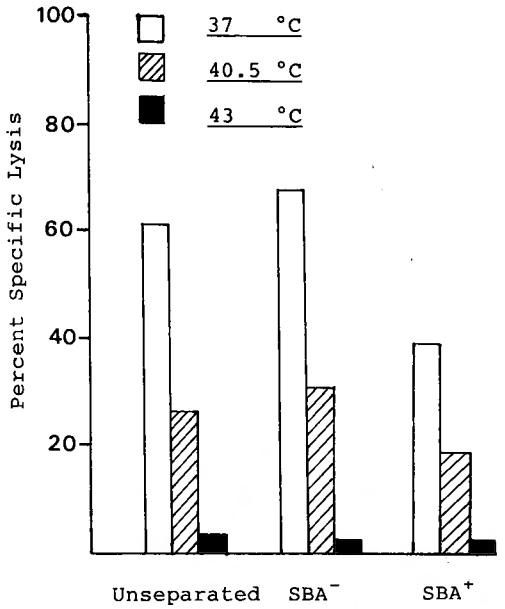

Fig. 3. NK activity of SBA-separated fractions after 2 h-incubation at 37°C, 40.5°C and 43°C.

The effect of heat on NK activity of human mononuclear cells was also reported[5]. AZOCAR and his colleagues reported that percent specific lysis was reduced when NK assay was performed at 40°C, and the effector cells, which had been incubated at 40°C before the NK assay at 37°C, lysed target cells much less effectively than effector cells incubated at 37°C

In this study we assayed NK activity of human MNC by [^{51}Cr] release assay for 4 h at 37°C after incubation for various durations at various temperatures. After incubation below 39°C, NK activity showed no change in spite of the incubation time. At 43°C, it disappeared almost completely within 1 hour. At 40.5°C it was reduced to about half by 1 h-incubation, but it did not exhibit further reduction and remained almost constant in spite of further incubation. At 40°C, it showed the same pattern as at 40.5°C in most cases; however, in a few cases it showed a small change. Therefore, we examined NK activity at 40.5°C. The first reduction and subsequent constant level of NK activity, when incubated at 40.5°C, indicates that there are two NK subpopulations which are distinguishable by temperature sensitivity; one subpopulation loses NK activity at 40.5°C and exhibits the first reduction phase, whereas the other is not affected and exhibits the subsequent constant phase (Figure 2).

As shown in table 3, NK activity of MNC, which had been first incubated at 37°C (control group), was reduced to about 50–80% by 20 h-incubation in 5% CO_2 incubator at 37°C (second incubation). NK activity was reduced more significantly when it was incubated at 4°C than when it was incubated at 37°C for 20 hours (data not shown). NK activity of MNC, which had been first incubated at 40.5°C, was reduced to about half, but it was not reduced after the second incubation and became almost the same as that in the control group. These phenomena show two possibilities. First, by 4 h-incubation at 40.5°C, a temperature sensitive subpopulation loses

NK activity, but it recovers NK activity from the effect of the temperature after 20 h-incubation in a 5% CO_2 atmosphere at 37°C. By 4 h-incubation at 43°C, MNC lose NK activity, but it is restored partially after the second incubation, although it is unclear which subpopulation (the heat sensitive or the resistant one) contributes the partial restoration. Second, the heat resistant (at 40.5°C) subpopulation also resists against in-vitro culture condition. Therefore it maintains the same NK activity after the second incubation as after the first incubation.

In the present study, we showed that the different sensitivity of NK cells to the temperature (40.5°C) is one of the properties which can separate NK cells into two subpopulations. These two subpopulations may overlap with the subpopulations separated by other properties or surface markers. Our SBA^+ fraction contained 55 to 88% (mean of five experiments, 67%) of the cells, but according to REISNER et al., it contained approximately 83% of the cells. They used autologous erythrocytes as carriers to maximize the efficiency of separation. However, we did not use autologous erythrocytes because the 0.83% ammonium chloride solution which is used to destroy erythrocytes reduced the NK activity of MNC (data not shown). Therefore, the observed difference of the proportion of SBA^+ and SBA^- fractions may be due to the difference in the separation procedure.

As shown in table 4, the SBA^- fraction showed stronger NK activity than the SBA^+ fraction. Then we examined the temperature sensitivity of each fraction. If the two fractions overlap with the two subpopulations which differed in temperature (40.5°C) sensitivity, one of the two fractions should lose NK activity completely and the other fraction should maintain NK activity after the incubation at 40.5°C. However, after a 2 h-incubation at 40.5°C, NK activity of both fractions was reduced to about half of the activity observed after incubation at 37°C (Figure 3). Therefore, the two fractions separated by SBA did not overlap with the two subpopulations separated by temperature sensitivity. It follows that human NK cells are heterogeneous.

Summary

Two NK subpopulations can be distinguished by temperature sensitivity; one subpopulation loses NK activity at 40.5°C, whereas the other is not affected. When we separated mononuclear cells with soybean agglutinin into agglutinated and unagglutinated fractions, the unagglutinated fraction showed stronger NK activity than the agglutinated fraction. These two fractions did not overlap with the two subpopulations which were distinguished by temperature sensitivity. Therefore, these results indicate that human NK cells are heterogeneous.

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和文抄録

人NK細胞のサブセット解析
—温度感受性と Soybean agglutinin による—

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NK細胞は，単一のものではなく，表面マーカーや，種々の特性により，いくつかの subpopulations に分けられている．我々は，ヒト NK 細胞が，温度感受性の差によっても，二つの subpopulations に分けられることを見出した．つまり，40.5°C で加温すると NK 活性を失う群と，40.5°C にても，NK 活性を失わない群とにである．

又，末梢単核球を，SBA にて，receptor の有無により，SBA^+ と SBA^- に分画し，両者の NK 活性を調べたところ，両者ともに，NK 活性を有するが，後者が，前者より高い活性を示した．

温度感受性の差，並に SBA receptor の有無により分けられた NK subpopulatios は，overlap せず，NK 細胞の heterogeneity を示している．