Orignal Works

Correlation Between Natural Killer Cell-Mediated Cytotoxicity and PBMC Differentiation in Healthy Volunteers

Jun Funaki

Inflammation and Immunology, Kyoto Prefectural University of Medicine*

Abstract: Assessing human immunity is important for preventing infections and cancer. Since the body's immune responses are complicated, it is necessary to assess immunity from different angles. In general, a person's resistance to cancer and infection is assessed by measuring natural killer (NK) cell-mediated cytotoxicity and the differentiation of immunocompetent cells obtained from a peripheral blood sample. The results of the present study show that accurate assessment of natural killer cell-mediated cytotoxicity depends on the test being done promptly after blood collection. This suggests that it is not appropriate to outsource natural killer cell-mediated cytotoxicity testing to outside laboratories. The differentiation of immunocompetent cells was analyzed using flowcytometry. It was found that CD4＋ and CD25＋ T cells and CD4/CD8 ratio have strong inverse correlations to natural killer cell-mediated cytotoxicity. Thus, it is useful to measure these two factors in order to assess human immunity.

Key Words: NK-mediated cytotoxicity, Human immunity, CD4/CD8, CD4CD25, PBMC.

Introduction

When antigens, such as, viruses and bacteria, enter the body, or when cancer cells are formed in the body, various biological reactions to eliminate the antigens occur[1)2)]; these biological reactions are called immune responses[3)4)], and the degree of the immune responses is generally referred to as resistance. Immunological resistance must be assessed from different angles. Resistance to cancer and infections can be assessed by investigating the differentiation[5)6)] and natural killer cell-mediated cytotoxicity of immunocompetent cells obtained from peripheral blood samples[7-11)]. In general, natural killer cell-mediated cytotoxicity is assessed by measuring the target cell cytotoxicity of peripheral blood mononuclear cells (PBMCs) that are obtained by centrifuging a blood sample. Since PBMCs are live cells, the natural killer cell-mediated cytotoxicity of PBMCs is thought to decrease with the length of time after blood collection. A highly reliable cytotoxicity test would include a thorough protocol,

automation of the testing procedures to minimize human errors, and proper handling of effectors and target cells to ensure accuracy. To assess differentiation of immunocompetent cells, monoclonal antibodies are used to stain cell surface markers for flowcytometry. Although natural killer cell-mediated cytotoxicity is an indicator of live cell function, the differentiation of immunocompetent cells is based on the recognition of cell surface markers. Thus, as long as the cells are alive, the test's accuracy is stable.

In this study, the length of time to isolate PBMCs after blood collection, which is the most important condition for the accurate measurement of natural killer cell-mediated cytotoxicity, was evaluated. Next, flowcytometry to determine the differentiation of immunocompetent cells was performed to assess natural killer cell-mediated cytotoxicity.

Materials & Methods

1．Reagents

All chemicals were of analytical grade. Labeling buffer contained 50 mM HEPES, 93 mM NaCl, 5 mM KCl, and 2 mM $MgCl_2$ in 1 liter of distilled water adjusted to pH 7.4. Repair buffer was prepared from labeling buffer by adding 2 mM $CaCl_2$ and 10 mM glucose. The stock solution of Eu^{3+} was prepared by dissolving 10 mM europium acetate (Wako Pure Chemical, Osaka, Japan) in saline. This stock solution was divided into aliquots and stored at $-20℃$. The DTPA stock solution was prepared by dissolving 100 mM DTPA in HEPES buffer (3.93 g in 100 ml) and adjusting the pH to 7.4 with 1.0 N NaOH. This solution was also divided into aliquots and stored at $-20℃$. RPMI 1640 medium supplemented with 10% fetal calf serum, $50 \mu g$ of streptomycin/ml, and 50 IU of penicillin/ml will be referred to as complete medium (CM). The antibodies against CD3, CD4, CD8, CD14, CD19, CD16, CD25, and CD56 were obtained from BD Pharmingen (San Jose, CA).

2．Purification of peripheral blood mononuclear cells

All healthy volunteers in this study provided written informed consent to participate. The protocol of the present study was approved by the Ethics Committee of Takeda Hospital Group (Kyoto, Japan). Blood samples were obtained in the morning from 40 healthy volunteers' ages 30-48 years and were diluted with phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). After centrifugation, PBMC were collected and washed three times in PBS, counted by Coulter Counter Z2 (Beckman Coulter, Fullerton, CA), and resuspended in CM. All PBMC designated for NK activity assays were stored overnight (18 h) at 37℃ in a 5% CO_2 incubator and tested the following morning. This procedure was implemented to equalize the time period between PBMC isolation and the assay.

Management of the time between the collection of blood and the isolation of PBMC from venous blood is the most crucial factor in the quality control of PBMC. To investigate this factor, blood collected from each of eight healthy volunteers was divided into six samples (5 mL each) and stored at room temperature, and PBMC was collected by density gradient centrifugation immediately after collecting blood and at 2, 5, 8, 11, and 24 hours after collecting blood.

For purposes other than the investigation of quality control, PBMC was collected by density gradient centrifugation immediately after collection of venous blood at rest. Furthermore, in another series of experiments on two healthy volunteers, PBMC was collected following blood collection immediately before, immediately after, and at 3, 6, and 24 hours after application of thermal stress,

which consisted of 60-min systemic heating using far infrared systemic heating equipment. Due to this thermal stress, the rectal temperature of subjects increased to 39°C at the end of systemic heating.

3. Target cells for cytotoxicity assays

K562, a human chronic myelogenous leukemia cell line, was maintained in culture in RPMI 1640 medium supplemented with 10% fetal bovine serum. The medium was changed three times a week and the cells were in the log phase of growth when used as targets in cytotoxicity assays.

4. Labeling of target cells with EuDTPA

About 5×10^6 target cells were washed in saline to reduce extracellular Ca^{2+} content. The cell pellet was resuspended in 1 ml of labeling buffer supplemented with $20 \mu M$ Eu^{3+}, $100 \mu M$ DTPA, and 0.5 mg of dextran sulfate. This cell suspension was incubated for 20 min at room temperature with occasional shaking. The labeling process was stopped by the addition of $30 \mu l$ of 100 mM $CaCl_2$ solution per ml. After 5 min of continued incubation, the cells were washed three times in 30 ml of repair buffer and were finally resuspended in CM at a concentration of 5×10^4 cells/ml.

5. Cytotoxicity assays

Target cells labeled with Eu^{3+} were adjusted to a concentration of 5×10^3 cells/$100 \mu l$ in CM. Aliquots $(100 \mu l)$ of target cells were dispensed into wells of 96-well round-bottomed microtiter plates. An equal volume of effector cells was added to each well. Suspensions of effector cells were adjusted to give effector/target （E/T） ratios ranging from 40：1 to 20：1 for NK activity. The microplates were centrifuged briefly to bring effectors and targets in contact with each other and then incubated for 4 h at 37°C. All assays were done in triplicate. After incubation, the plates were centrifuged again and the supernatants were harvested for measurements of released Eu^{3+}. For the detection of released Eu^{3+}, $20-\mu l$ aliquots of the supernatants were transferred to wells of a flat-bottom 96-well microplate and a $200-\mu l$ aliquot of enhancement solution was added to each well. After mixing for 5 min, fluorescence was measured in a time-resolved fluorometer （Wallac, Waltham, MA）. The percentage of specific cytotoxicity was calculated as （experimental release-spontaneous release）/（maximum release-spontaneous release）$\times 100$.

Spontaneous release was determined by incubating the targets with 100 ml of CM instead of effector cells and maximum release was determined by incubating the targets with 100 ml of 0.5% Triton-X.

6. Flow cytometric analysis of PBMC

Samples were stained with fluorescently labeled antibodies to CD3, CD4, CD8, CD14, CD19, CD16·CD56, CD3·CD16·CD56, and CD4·CD25. All staining was performed in PBS, 2.5% FCS, and 5 mM EDTA. All flow cytometry was performed on a fluorescence activated cell sorter （BD FACSCalibur, BD, Franklin Lakes, NJ）.

7. Statistical analysis

To analyze differences in levels of cytotoxicity between groups of individuals, Scheffe multiple comparison test was used and differences were considered significant when P values were less than 0.05. An analysis of the correlations between the cytotoxicity and each factor of PBMC was carried out using Stat View 5.0 software on a Macintosh computer （Abacus Concepts Inc., Berkeley, CA）.

Table 1. Natural killer cell-mediated ytotoxicity of 8 volunteers. The time indicated the length of time from blood collection to PBMC isolation

| No	0 h (% of 0 h)	2 h	5 h	8 h	11 h	24 h
1	44.7 (100)	42.4 (94.9)	39.1 (87.5)	37.8 (84.6)	37.5 (83.9)	25.5 (57.0)
2	43.6 (100)	39.3 (90.1)	36.0 (82.6)	30.9 (70.9)	31.1 (71.3)	26.9 (61.7)
3	40.8 (100)	36.7 (90.0)	34.9 (85.5)	30.1 (73.8)	31.8 (77.9)	24.0 (58.8)
4	33.4 (100)	29.7 (88.9)	29.0 (86.8)	23.5 (70.4)	26.0 (77.8)	22.5 (67.4)
5	22.6 (100)	22.4 (99.1)	22.1 (97.8)	19.0 (84.1)	20.7 (91.6)	17.1 (75.7)
6	36.0 (100)	29.8 (82.8)	29.6 (82.2)	26.4 (73.3)	25.4 (70.6)	17.0 (47.2)
7	37.1 (100)	27.7 (74.7)	27.6 (74.4)	25.3 (68.2)	25.9 (69.8)	16.3 (43.9)
8	63.5 (100)	61.0 (96.1)	59.2 (93.2)	54.5 (85.8)	46.5 (73.2)	37.7 (59.4)
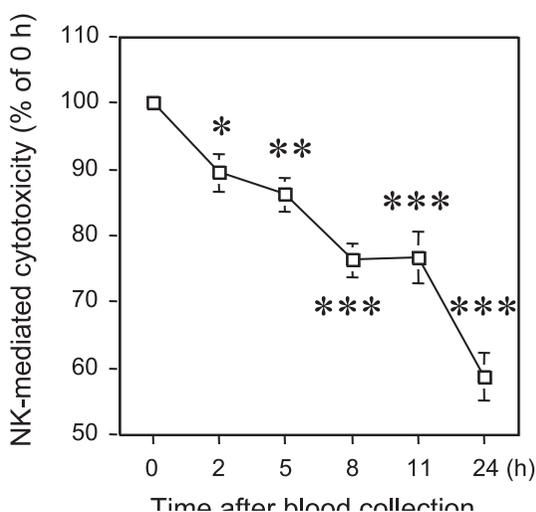
Fig. 1. Effects of the length of time from blood collection on NK-mediated cytotoxicity.
Values are the means ± SEM of 8 samples. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs 0 h.

Results

1. PBMC accuracy

The relationship between natural killer cell-mediated cytotoxicity and the length of time from blood collection to PBMC isolation was investigated. As shown in Table 1 and Fig. 1, natural killer cell-mediated cytotoxicity decreased as the length of time from venous blood collection to PBMC isolation increased. Compared to when PBMCs were isolated immediately after blood collection, natural killer cell-mediated cytotoxicity decreased by 10.4% when a venous blood sample was left standing for 2 hours at room temperature and by 43.8% when a venous blood sample was left standing for 24 hours.

2. Correlation between natural killer cell-mediated cytotoxicity and PBMC differentiation

Table 2 shows the immunity (natural killer cell-mediated cytotoxicity and PBMC differentiation) at the time of blood collection of 30 healthy volunteers. Based on the 11 items shown in the table,

Table 2. The immunity（natural killer cell-mediated cytotoxicity and PBMC differentiation）of 30 heathy volunteers.

No.	NK activity (%)		CD3	CD4	CD8	CD14	CD19	NKT	NK	CD4+CD25+	CD4:CD8
	40:1	20:1									
1	22.5	12.3	69.10	52.43	16.76	6.82	10.93	0.93	15.70	28.30	0.55
2	21.3	12.2	60.36	42.08	13.22	9.93	8.49	0.53	19.32	24.89	0.78
3	45.2	24.5	52.39	41.85	20.78	16.83	9.38	0.90	25.64	25.95	0.99
4	33.3	14.2	65.65	39.86	29.63	7.97	8.73	3.07	24.78	19.97	1.24
5	24.3	10.2	65.76	42.25	16.72	7.18	6.15	2.88	20.50	23.23	0.88
6	31.4	15.9	49.81	31.95	31.39	27.22	7.21	4.18	35.22	16.81	2.10
7	36.9	17.8	66.13	38.39	23.72	5.83	5.39	2.36	24.41	19.07	1.28
8	24.6	11.0	63.38	37.00	20.98	11.51	7.42	2.44	23.00	18.25	1.26
9	36.7	19.7	54.77	32.68	30.43	12.11	5.91	2.73	32.81	15.90	2.06
10	11.8	4.0	81.07	54.86	17.18	4.73	7.42	1.69	7.16	24.27	0.30
11	7.8	4.0	79.00	54.59	15.66	8.60	10.26	1.41	6.80	27.94	0.24
12	10.4	4.3	75.83	55.53	21.63	21.99	7.76	1.52	8.40	27.27	0.31
13	19.0	6.3	75.39	51.50	25.35	6.91	5.82	1.76	14.36	20.45	0.70
14	13.1	4.6	79.55	54.41	24.99	14.21	7.71	1.69	10.29	24.05	0.43
15	16.7	6.1	76.24	51.69	27.31	17.68	8.65	1.54	11.94	20.67	0.53
16	27.4	11.1	84.95	60.57	24.40	4.13	5.46	1.06	8.66	21.61	0.40
17	16.4	6.7	82.83	56.42	26.82	5.91	8.93	1.04	8.19	22.58	0.36
18	31.3	17.2	66.83	47.35	29.90	12.29	6.40	1.69	24.53	16.77	1.46
19	11.1	6.0	67.58	35.61	27.75	10.51	10.80	1.00	12.15	14.59	0.83
20	10.2	4.3	75.25	39.59	24.25	8.54	13.31	1.44	9.96	16.40	0.61
21	20.6	8.3	61.77	31.35	30.35	12.76	10.47	1.20	19.80	11.38	1.74
22	28.1	11.7	70.21	42.71	27.83	10.10	5.97	1.98	18.35	22.78	0.81
23	23.3	8.9	64.21	41.10	17.77	5.44	4.15	1.57	24.51	21.60	1.13
24	31.0	13.7	60.88	36.57	27.60	13.41	6.33	1.94	24.95	19.05	1.31
25	28.0	15.4	60.95	28.01	33.89	8.83	7.96	1.39	25.24	11.99	2.11
26	24.3	10.8	76.75	32.80	24.69	5.47	5.75	1.16	16.06	13.59	1.18
27	16.4	5.3	59.27	25.33	25.27	14.54	10.95	1.56	16.53	9.82	1.68
28	7.1	3.0	69.15	50.59	16.62	8.43	10.45	1.83	11.03	29.59	0.37
29	9.2	2.8	70.44	44.12	12.84	4.49	4.61	2.19	17.61	25.43	0.69
30	12.2	6.1	59.42	44.74	18.91	13.63	7.72	3.21	23.05	26.00	0.89

8 figures were drawn; natural killer cell-mediated cytotoxicity（40：1）was plotted on the Y-axis, while the remaining 8 factors were plotted on the X-axis（Fig. 2A through 2H）. Of the 8 factors, CD4＋CD25＋ T cells and CD4/CD8 ratio were the two factors with a very close correlation coefficient（｜R｜＞0.7）. Furthermore, correlation coefficient between CD4＋CD25＋ T cells and CD4/CD8 ratio showed a significant correlation （｜R｜＝0.87）.（Fig. 3）

3．*The correlation between the differentiation of immunocompetent cells and changes in natural killer cell-mediated cytotoxicity related to thermal stress*

The results showed that thermal stress altered natural killer cell-mediated cytotoxicity. Following thermal stress, CD4＋CD25＋ T cells and CD4/CD8 levels fluctuated in response to the changes in natural killer cell-mediated cytotoxicity. Both CD4＋CD25＋ T cells and CD4/CD8 levels had a marked inverse correlation to natural killer cell-mediated cytotoxicity（Fig. 4A, 4B, 5A and 5B）. The correlation
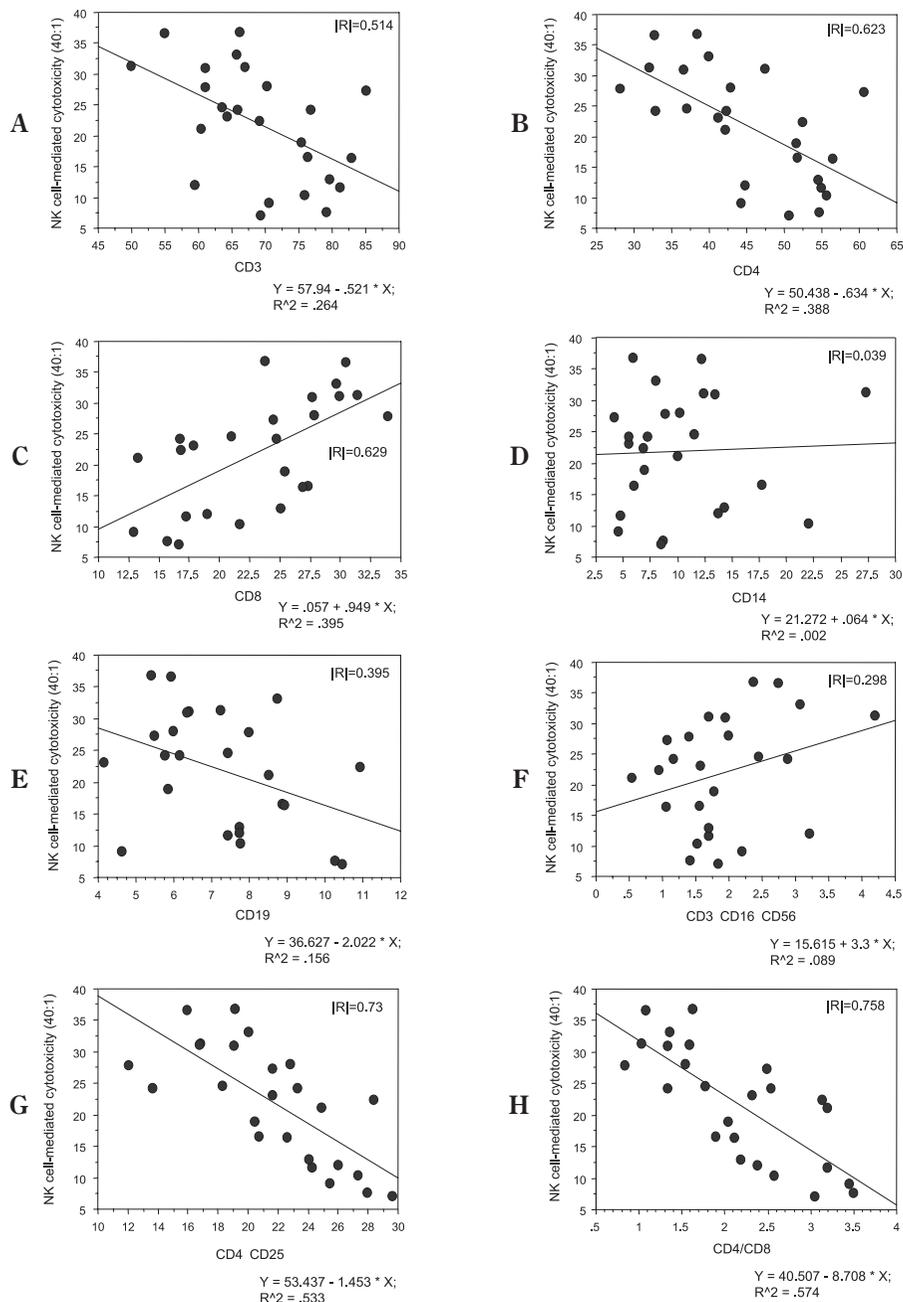
Fig. 2.   Correlation between NK-mediated cytotoxicity and PBMC differentiation
A）CD3 vs NK-cytotoxicity:｜R｜＝0.514, p＝0.0085,
B）CD4 vs NK-cytotoxicity:｜R｜＝0.623, p＝0.0009,
C）CD8 vs NK-cytotoxicity:｜R｜＝0.629, p＝0.008,
D）CD14 vs NK-cytotoxicity:｜R｜＝0.039, p＝0.852,
E）CD19 vs NK-cytotoxicity:｜R｜＝0.395, p＝0.051,
F）NKT vs NK-cytotoxicity:｜R｜＝0.298, p＝0.149,
G）CD4CD25 vs NK-cytotoxicity:｜R｜＝0.73, p＜0.0001,
H）CD4/CD8 vs NK-cytotoxicity:｜R｜＝0.758, p＜0.0001

$$Y = -1.133 + .151 * X;$$
$$R^2 = .757$$
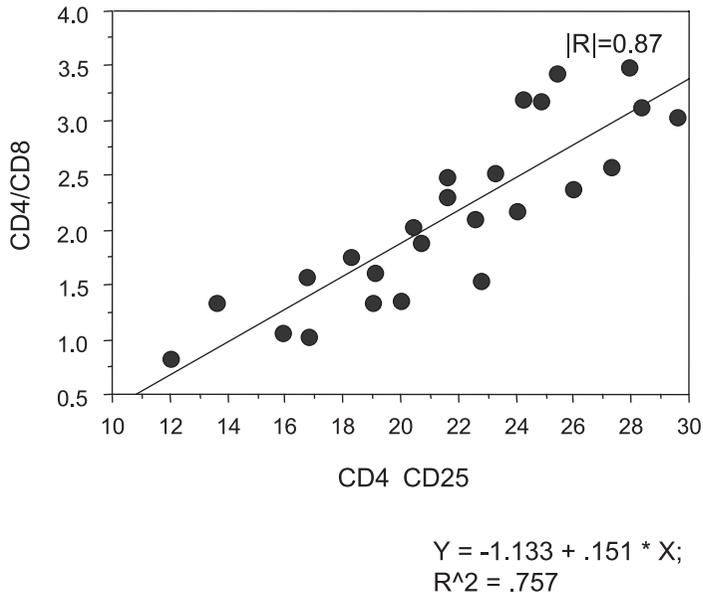
Fig. 3.　Correlation coefficient between CD4+CD25+ T cells and CD4/CD8 ratio.
｜R｜=0.87, p<0.0001

$$Y = 28.284 - 4.983 * X;$$
$$R^2 = .953$$

A

$$Y = 71.199 - 27.264 * X;$$
$$R^2 = .987$$
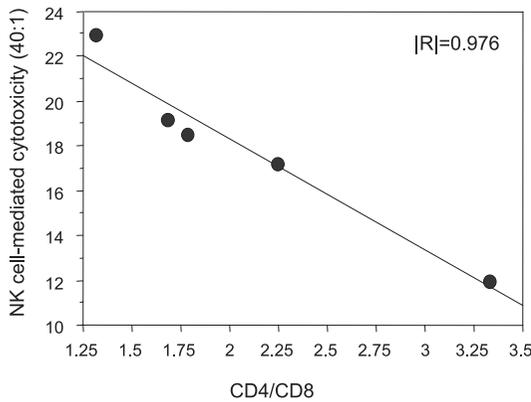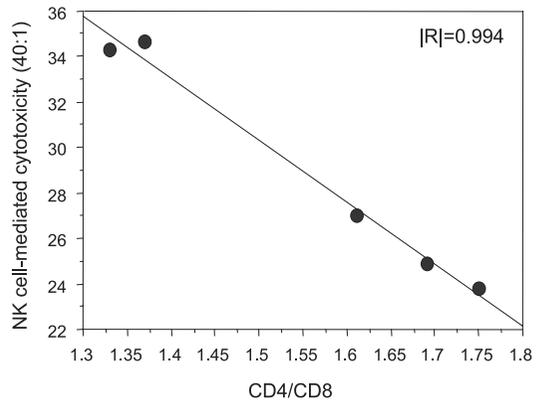
B

Fig. 4.　Correlation between NK-mediated cytotoxicity and CD4/CD8 after thermal stress.
　　　Thermal stress induced the transient down-regulation of NK cell activity. Therefore we measured the NK-mediated cytotoxicity after thermal stress from two volunteers.
　　　A）NK-mediated cytotoxicity of a certain volunteer. PBMC was collected following blood collection immediately before, immediately after, and at 3, 6 and 24 hours after thermal stress. CD4/CD8 vs NK-cytotoxicity: ｜R｜=0.976, p<0.0001,
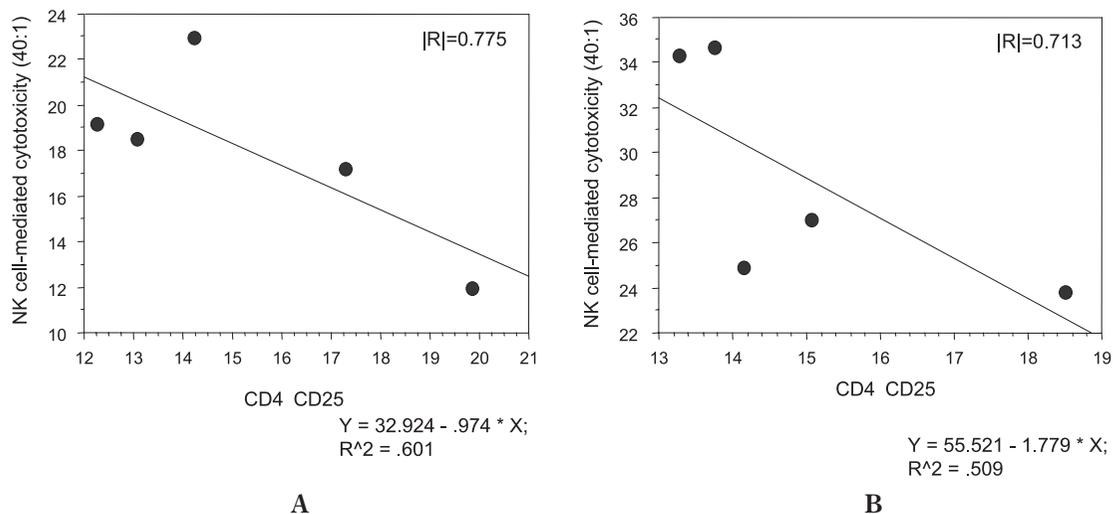　　　B）NK-mediated cytotoxicity of other volunteer. ｜R｜= 0.994, p<0.0001.

Fig. 5. Correlation between NK-mediated cytotoxicity and CD4CD25 after thermal stress.
  A）NK-mediated cytotoxicity of a certain volunteer. PBMC was collected following blood collection immediately
      before, immediately after, and at 3, 6 and 24 hours after thermal stress. CD4CD25 vs NK-cytotoxicity: ｜R｜＝
      0.775, p＜0.0001,
  B）NK-mediated cytotoxicity of other volunteer. ｜R｜＝0.713, p＜0.001.

coefficient（R）of natural killer cell-mediated cytotoxicity to CD4/CD8 was≧0.97; this suggests a very close relationship.

Discussion

Cytotoxicity tests are an integral part of assessing human immunity to ascertain the function of live cells[1)12)]. The conditions under which PBMCs are collected markedly affect the test results. The first goal of the present study was to clarify the conditions necessary for proper PBMC management to ensure the accurate assessment of natural killer cell-mediated cytotoxicity. One of the factors related to test accuracy is the length of time from venous blood collection to PBMC isolation. The results of the present study showed that, to accurately measure natural killer cell-mediated cytotoxicity at the time of blood collection, the PBMCs must be promptly isolated from the blood sample. Therefore, in the present study, PBMCs were immediately isolated after blood collection for subsequent experiments.

However, most hospitals and clinics do not perform in-house cytotoxicity tests, such as, natural killer cell-mediated cytotoxicity assay, but outsource these tests to outside laboratories. Therefore, it is difficult to ensure that the length of time from blood collection to the time that cytotoxicity testing is done is consistent; in fact, in some cases, cytotoxicity tests may be done more than 24 hours after blood collection, which leads to inaccurate results. However, when assessing human immunity, testing cytotoxicity, such as natural killer cell-mediated cytotoxicity, is very important.[1)3)4)] The second goal of the present study was to identify a parameter that could be assessed in blood samples left standing at room temperature that would accurately reflect natural killer cell-mediated cytotoxicity.

When using flowcytometry, special handling of the samples is not necessary to achieve stable

measurements of the expression of various surface antigen molecules. The present study focused on CD4/CD8 and CD4＋CD25＋ T cells; the results showed that the cytotoxicity of immunocompetent cells acquired from healthy volunteers could be assessed without the need to directly measure natural killer cell-mediated cytotoxicity. On the other hand, it was reported that CD4＋CD25＋ Tcell suppressed NK cell-mediated cytotoxicity, and it supports the results of this study.[13)-15)] Of note, within the same individual, changes in natural killer cell-mediated cytotoxicity could be accurately assessed based on changes in CD4/CD8 levels.

Thus, it is meaningless to investigate human immunity by measuring natural killer cell-mediated cytotoxicity in improperly handled samples in which the PBMCs have not been isolated promptly. In such a case, it is important to measure CD4/CD8 and CD4＋CD25＋ T cells by flowcytometry.

In addition, thermal stress exerts an influence on NK cell-mediated cytotoxicity, and the effects have various patterns. For instance, sometimes it enhances NK cell-mediated cytotoxicity, but sometimes it down-regulates.[16)] This effect was also observed in this study. Even though thermal stress enhanced or suppressed NK cell-mediated cytotoxicity, measuring CD4/CD8 and CD4＋CD25＋ T cells by flowcytometry was comparable with assessing NK cell-mediated cytotoxicity. When monitoring immunity over time in comprehensive health screenings, CD4/CD8 and CD4＋CD25＋ T cells are particularly useful for ascertaining immunological changes in the same healthy individual.

Acknowledgements

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〈和文抄録〉

健常人における NK 活性と PBMC 分画の相関についての検討

舟　木　　　準

京都府立医科大学大学院医学研究科生体機能制御学

感染や癌を予防するにあたり，生体の免疫能を測定することは重要であるが免疫機構は非常に複雑であるため，免疫能を評価するためには様々な方面からのアプローチが必要となる．ただし，一般的には感染や癌に対する免疫能を評価する場合は末梢血単核球由来のナチュラルキラー（NK）細胞の細胞障害活性を測定することが多い．本研究でこのNK活性が採血後の時間経過とともに低下することが明らかになったため，NK 活性を正確に算出するためには，採血後速やかに測定を始めることが重要であると考えられる．したがって，測定開始までに時間を要する外注検査には適していないと考えられる．一方，免疫に関わる細胞分類はフローサイトメトリーを用いて評価できるが，CD4＋/CD25＋T 細胞と CD4/CD8 比は NK 活性と強い負の相関関係を有することが解ったため，これらの 2 つのパラメータを測定すればNK 活性を評価することが可能であると考えられる．

キーワード：免疫能，NK 活性，CD4＋/CD25＋ T 細胞，CD4/CD8 比.