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KEYWORDS: ADCC acitivity, NK activity, Thy 1 antigen, plastic dish fractionation, tumor-bearing mice

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DISSOCIATION OF NATURAL KILLER ACTIVITY AND ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN SPLEEN CELLS OF TUMOR BEARING MICE

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Abstract. Antibody-dependent cellular cytotoxicity (ADCC) increased with the development of tumors in C3H/He mice bearing spontaneous breast cancer or the syngeneic hepatoma MH-134 and in C57BL/6 mice bearing the syngeneic Lewis lung carcinoma 3LL. This cytotoxicity decreased after treatment with guinea pig, monoclonal IgM anti-Thy 1.2 serum and complement to the non-cancer level thus indicating that the increased ADCC in mice with cancer seems mainly attributable to cells with the Thy 1 antigen. On the other hand, NK activity decreased greatly when mice had tumors. Treatment with monoclonal IgM anti-Thy 1.2 serum and complement showed no significant influence on the natural killer (NK) activity of spleen cells of mice bearing MH-134 cancer, but in the 3LL-bearing mice the activity decreased significantly.

Key words : ADCC activity, NK activity, Thy 1 antigen, plastic dish fractionation, tumor-bearing mice.

There have been many reports recently on natural killer (NK) activity and antibody-dependent cellular cytotoxicity (ADCC). The effector cells of these activities, NK cells and K cells, both possess IgG-Fc receptors which have been suggested to be the same or nearly the same, though this hypothesis has not been clearly demonstrated. NK activity increases in the early period of tumor bearing, and is supposed to decrease thereafter. However, there are few discussions on its relation to ADCC.

We have observed that there is a high correlation between ADCC and NK activity in healthy donors, and that while this correlation was less in cancer patients, there was a high correlation between ADCC activity and the percentage of IgG-Fc receptor positive T cells in lymphocytes from cancer patients (1). In the present study, ADCC and NK activity of murine spleen cells are compared in mice with and without tumors by fractionating the subpopulations of the lymphocytes.

MATERIALS AND METHODS

Mice and Tumors. Male and female inbred strains of C3H/He and (C57BL/6 × BALB/c) F_1 (CBF_1) mice between 8 and 12 weeks of age were supplied by the Okayama University

Medical School.

MH-134 ascites hepatoma cells obtained from C3H/He mice and Lewis lung carcinoma (3LL) cells obtained from C57BL/6 mice, were inoculated (1×10^6 cells/0.1 ml) subcutaneously into the back of mice to induce tumors in mice. In addition, C3H/He mice with spontaneous breast cancer were used.

Preparation of effector cells. The spleen was excised, cut into small pieces in culture medium, filtered through # 150 mesh, erythrocytes destroyed with 0.75 % NH_4Cl-tris buffer (pH 7.65), centrifuged at 1000 rpm for 5 min, and washed twice with phosphate buffer saline (PBS) and once with culture medium solution. The washed cells were transferred to a plastic dish (diameter 9 cm), incubated at 37 ℃ under 5 % CO_2 for 1 h in medium, and then adherent cells were separated from floating cells. The same procedure was repeated 3 times to separate adherent cells from floating cells obtaining a final monocyte content of under 1 % of the floating cells.

Preparation of target cells. Chicken red blood cells (CRBC) were used as target cells for the ADCC assay. Blood was taken, washed twice with PBS and once with culture medium, and then 200 μCi of $Na_2{}^{51}CrO_4$ (Kaken Chemical Co. Ltd.) was added to each ml of the suspension containing 1×10^7 CRBC. The cells were labeled at 37 ℃ under 5 % CO_2 for 1 h, after which they were washed 3 times with culture medium.

As target cells for the NK assay, fetal fibroblasts and measles-virus infected HeLa cells (M-HeLa) were used. The fetal lung of RF mice was treated at 37 ℃ with 0.125 % trypsin (DIFCO Laboratories, USA) and 0.05 % collagenase (SIGMA Chemical Co., USA) to obtain fibroblasts which were poured into wells of a Falcon 3042 microtest II type plate (Falcon Plastics, USA) at 1×10^4 cells/well. The fibroblasts were incubated at 37 ℃ under 5 % CO_2 for 12 h, and then after addition of 2 μCi/well of $Na_2{}^{51}CrO_4$ for an additional 6 h. Also to use as target cells for the NK assay, 3LL primary culture cells were obtained from tumor pieces incubated with 0.25 % trypsin-Hanks buffer without Ca^{2+} and Mg^{2+} ions at 37 ℃ for 15 min. The cells were adjusted to 1×10^6 cells/ml in culture medium, incubated with 200 μCi of $Na_2{}^{51}CrO_4$ at 37 ℃ under 5 % CO_2 for 1 h, and suspended in culture medium after washing.

Antiserum. A 10 % CRBC solution was mixed with Freund's complete adjuvant, and inoculated at several places intradermally on both feet once a week for 5 weeks. Blood was taken 1 week after the final inoculation, and the serum was separated and inactivated by heating at 56 ℃ for 30 min. The agglutination titer was measured, and from the minimum agglutination titer the optimum concentration was determined to be 1 : 5000.

ADCC assay. The details have already been reported (2) but briefly, 1×10^5 ^{51}Cr-labeled CRBC 100 μl of medium were placed in each well ; 50 μl of rabbit anti-CRBC antibody was added, and the mixture was incubated at 37 ℃ for 30 min. Then, effector cells 5×10^5 cells/100 μl were added at an effector cell to target cell (E : T) ratio of 5 : 1 and incubated at 37 ℃ under 5 % CO_2 for 12 h. The cells were centrifuged at 1000 rpm for 3 min ; the supernatant was removed and radioactivity was measured with a γ-spectrometer (experimental release). As a control, the reaction was allowed to take place without the addition of effector cells (spontaneous release). From the C3H/He mice with spontaneous breast cancer, the tumor and the spleen were removed, and the wet weight of the tumor and the ADCC of the spleen cells were measured.

NK assay. To each kind of target cell (fetal mouse lung fibroblast, M-HeLa and 3LL) labeled with ^{51}Cr, effector cells were added to an E : T ratio of 100 : 1 and incubated as in the ADCC assay, after which radioactivity was measured (experimental relase). As a control,

only culture medium was added (spontaneous release).

Total release was obtained by destroying the cells with IN NaOH, and % specific lysis was calculated with the following formula :

$$\% \text{ Specific lysis} = \frac{\text{Mean experimental release - mean spontaneous release}}{\text{Mean total release - mean spontaneous release}} \times 100$$

All the experiments were conducted in triplicate, and the mean values were calculated.

Anti-Thy 1.2 serum treatment. Nonadherent cells were incubated for 30 min at 4 ℃ with a 1 : 1000 final dilution of monoclonal IgM anti-Thy 1.2 serum (Olac) derived from a hybridoma washed, and then centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended with a 1 : 4 dilution of dried guinea pig complement (C) (Kyokuto Seiyaku Industry Co. Ltd.) for 40 min at 37 ℃. As a control, only the complement was used. The surviving cells were washed 3 times, and viable cells were counted by the trypan blue dye exclusion method. The rate of reduction of ADCC and NK activity by the elimination of the T cells was calculated with the following formula :

$$\% \text{ Reduction} = 100 - \frac{\% \text{ cytotoxicity of cells treated with anti-Thy 1 plus C}}{\% \text{ cytotoxicity of cells treated with C only}} \times 100$$

Culture medium. The medium used was Roswell Park Memorial Institute 1640 tissue culture medium (RPMI 1640) (Nissui Seiyaku Co. Ltd.) with 25 mM N'-2-hydroxy ethylpiperazine N'-2-ethane sulfonic acid (HEPES) buffer solution (Sigma Chemical Co., USA), containing 100 U/ml crystalline penicillin G potassium, 100 μg/ml streptomycin sulfate (Meiji Seika Co. Ltd.), and 10 % heat-inactivated fetal bovine serum (FBS) (Grand Island Biological CO., USA).

RESULTS

ADCC of mice with spontaneous breast cancer. The ADCC did not change until the tumor weight reached 0.2 g, but thereafter, the activity increased with the development of the tumor, and once the wet tumor weight reached 1.4 g, the ADCC activity decreased (Fig. 1).

ADCC of MH-134 tumor bearing mice. The ADCC of unfractionated spleen cells of mice with MH-134 cancer increased significantly 5 days after tumor transplantation, and reached a peak around the 9th to 14th day. This high activity lasted until about the 20th day.

The adherent cell fraction had less activity than the unfractionated cells and the activity from the 5th to the 20th day after tumor inoculation was about 10 % of that of the group without cancer.

In the nonadherent cell fraction of the group with cancer, a higher activity was seen than in the adherent cell fraction, and when this nonadherent cell fraction was treated with anti-Thy 1.2 IgM serum and complement to remove T cells, the activity decreased markedly from 57.8 ± 3.8 % to 34.6 ± 1.5 % at the 9th day after tumor inoculation (Table 1, Fig. 2). The group without cancer showed a reduction of only 7.0 ± 5.6 %, while the group with cancer showed a reduction in activity of 26.3 ± 10.3 % on the 3rd day and 40.1 ± 7.9 % on the 9th day.

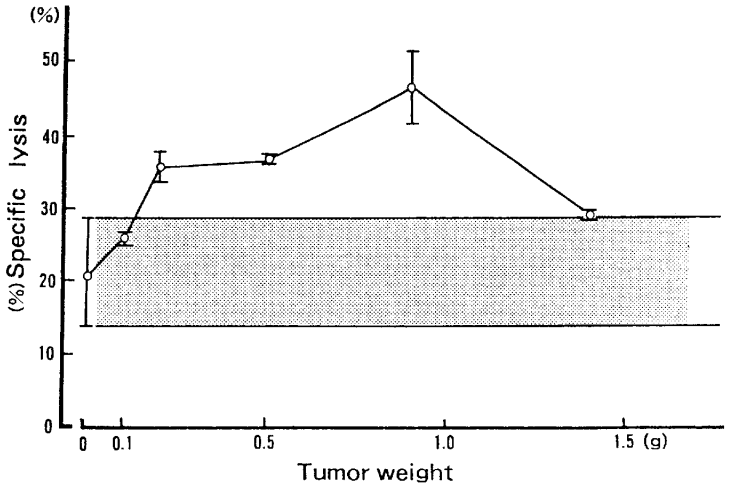

Fig. 1. Correlation between the ADCC of spleen cells and development of tumors in mice with spontaneous breast cancer. ^{51}Cr-labeled CRBC target cells (10^5/well) were combined with 5×10^5 spleen cells in a 12 h chromium-51 release assay. The spleen cells were from spontaneous breast cancer-bearing mice (o—o) or normal mice (▨). The specific lysis in the normal control was 21.5 ± 7.6 % for CRBC. Significance of the difference from normal spleen cells when the tumor weight was 0.2 g, $p < 0.05$; 0.5 g, $p < 0.01$ and 0.9 g, $p < 0.001$ (Student's t-test).

TABLE 1. COMPARISON OF ADCC OF THE SPLEEN CELL FRACTIONS FROM MH-134 TUMOR BEARING MICE

Fraction	Days after MH-134 tumor transplantation					
	0	3	5	9	14	20
Unfractionated	25.5± 10.5	31.3± 5.4	46.5± 8.1	50.6± 1.2	52.0± 1.6	49.8± 6.6
Adherent	19.4± 4.6	20.9± 2.9	29.7± 4.5	27.6± 2.7	28.2± 2.7	28.7± 3.2
Nonadherent	32.8± 6.5	39.5± 7.3	52.1± 6.1	57.8± 3.8	56.4± 1.8	53.0± 5.1
Anti-Thy 1+C	30.5± 6.1	29.1± 3.2	35.7± 3.1	34.6± 1.5	37.6± 1.0	33.2± 2.6

The effector to target ratio was 5 : 1. The values are expressed as the mean ± S.D.

This tendency of increased reduction after anti-Thy 1 treatment was noted until the 20th day (Table 2).

ADCC in mice with 3LL cancer. The ADCC in mice with 3LL cancer changed similarly as in mice with MH-134 cancer. Namely, the ADCC of mice with 3LL cancer increased with the development of tumors and reached a peak 10 days after tumor transplantation. The activity of the nonadherent cell fraction was higher than the adherent cell fraction. When T cells were removed by treatment with anti-Thy 1.2 serum and complement (Table 3), the activity decreased to the normal level and the % reduction was higher than that of the normal group (Table 2).

NK activity of mice with MH-134 cancer. The NK activity of the spleen cells

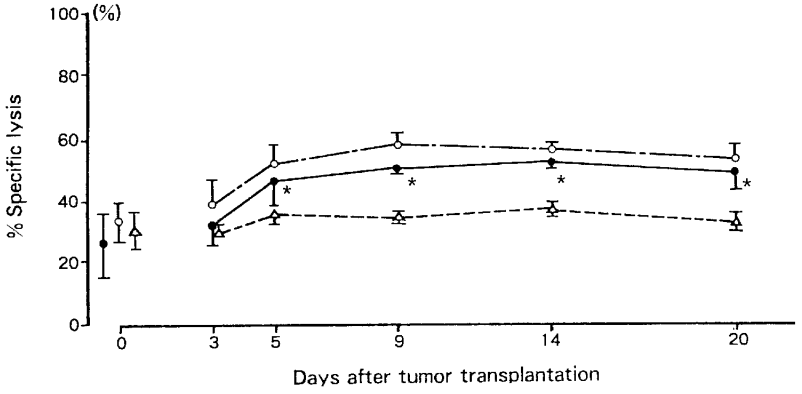

Fig. 2. Changes in ADCC of spleen cells from MH-134 tumor bearing mice. ^{51}Cr-labeles CRBC target cells (10^5/well)were combined with 5×10^5 spleen cells from MH-134 tumor bearing mice in a 12-h chromium-51 release assay. Unfractionated spleen cells (●—●), nonadherent cells (○-○), and anti-Thy 1.2 serum + C (△-△). *Significantly different from normal spleen cells (Day 0), $p < 0.01$ (Student's t-test).

TABLE 2. THE EFFECT OF ANTI-Thy 1.2 PLUS C ON THE ADCC OF SPLEEN CELLS FROM MH-134 OR 3LL TUMOR BEARING MICE

Effector cells	% Reduction by treatment with anti-Thy 1 plus C[a]									
	Days after tumor transplantation									
	0	1	3	5	9	10	14	15	18	20
MH-134 bearing mice	7.0±5.6		26.3±10.3	31.5±8.8	40.1±7.9		33.3±6.3			29.5±11.2
3LL bearing mice	15.9±7.5	-7.7±6.0	35.7±9.1	38.3±6.7		51.6±11.7		37.4±8.9	20.4±9.8	

[a] Percent reduction in lysis was calculated as described in Materials and Methods.
The effector to target ratio was 5 : 1 in all instances. The values are expressed as the mean±S.D.

TABLE 3. COMPARISON OF ADCC OF THE SPLEEN CELL FRACTIONS FROM 3LL TUMOR BEARING MICE

Fraction	Days after 3LL tumor transplantation						
	0	1	3	5	10	15	18
Unfractionated	24.5±4.4	27.8±3.8	30.6±4.6	31.9±3.2	35.1±2.9	29.4±3.5	20.0±4.8
Adherent	21.0±3.5	21.5±3.3	20.3±4.7	20.6±5.2	22.3±3.3	18.8±3.4	15.1±4.0
Nonadherent	27.5±4.9	30.0±5.0	36.7±3.8	36.8±4.1	43.2±4.2	35.3±4.1	23.5±3.7
Anit-Thy 1+C	23.1±2.4	32.3±3.4	23.6±3.2	22.7±3.3	20.9±3.0	22.1±3.5	18.7±3.5

The effector to target ratio was 5 : 1. The values are expressed as the mean±S.D.

of normal C3H/He mice to fetal lung fibroblasts of RF mice was seen in both the adherent and nonadherent cell fractions. The activity of spleen cells of mice with MH-134 decreased after tumor transplantation in both the adherent and

TABLE 4. COMPARISON OF NK ACTIVITY OF SPLEEN CELL FRACTIONS FROM MH-134 TUMOR BEARING MICE AGAINST FETAL FIBROBLAST TARGET CELLS

Fraction	Days after tumor transplantation			
	0	5	9	14
Unfractionated	14.8± 2.6	10.0± 2.8	7.8± 5.0	4.7± 4.9
Adherent	19.0± 2.5	9.6± 4.1	10.4± 3.8	6.2± 5.8
Nonadherent	21.3± 6.3	12.1± 5.6	11.9± 2.6	7.3± 3.8
Anti-Thy 1+C	17.9± 4.7	8.8± 4.7	8.4± 1.6	5.8± 1.4

The target cells were fetal fibroblasts. 1×10^4 ^{51}Cr-labeled fetal fibroblasts were mixed with 1×10^6 fractionated spleen cells (E/T ratio of 100 : 1). The values are expressed as the mean± S.D.

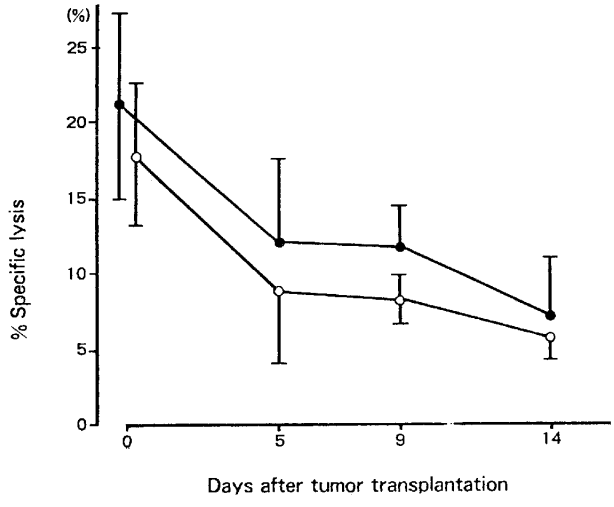

Fig. 3. Changes in NK activity of spleen cells from MH-134 tumor bearing mice. ^{51}Cr-labeled fetal fibroblasts (10^4/well) were combined with 10×10^5 spleen cells from MH-134 tumor bearing mice in a 12-h chromium-51 release assay. Nonadherent cell (•—•) and anti-Thy 1.2 serum + C (○—○).

TABLE 5. COMPARISON OF NK ACTIVITY OF SPLEEN CELL FRACTIONS FROM MH-134 TUMOR BEARING MICE AGAINST M-HeLa TARGET CELLS

Fraction	Days after tumor transplantation					
	0	3	7	10	14	17
Unfractionated	15.7± 2.3	12.8± 4.0	11.0± 2.8	7.0± 2.4	5.5± 4.8	0.4± 3.6
Adherent	12.1± 2.4	12.1± 3.0	11.5± 3.3	7.2± 3.1	7.6± 4.2	N.T.
Nonadherent	13.9± 2.7	13.6± 4.1	12.4± 3.0	9.0± 2.7	9.4± 3.5	N.T.
Anti-Thy 1+C	12.6± 3.4	11.1± 3.0	9.0± 4.2	6.3± 4.4	7.3± 3.8	N.T.

The target cells were M-HeLa cells. 1×10^4 ^{51}Cr-labeled M-HeLa cells were mixed with 1×10^6 fractionated spleen cells (E/T ratio of 100 : 1). N.T.: not tested. The values are expressed as the mean ± S.D.

TABLE 6. COMPARISON OF NK ACTIVITY OF SPLEEN CELL FRACTIONS FROM 3LL TUMOR BEARING MICE AGAINST M-HeLa TARGET CELLS.

Fraction	Days after tumor transplantation						
	0	1	3	5	10	15	18
Unfractionated	25.3± 1.6	23.2± 3.4	23.0± 3.5	21.8± 2.9	16.1± 3.5	14.0± 4.5	8.4± 5.2
Adherent	21.8± 2.2	21.1± 2.5	21.4± 3.2	20.3± 3.0	17.4± 3.6	17.2± 4.2	7.7± 4.6
Nonadherent	26.0± 2.8	24.5± 2.0	24.5± 2.3	22.9± 3.7	18.2± 3.3	18.9± 3.9	12.3± 4.4
Anti-Thy 1+C	23.1± 2.0	21.9± 3.3	18.4± 3.6	15.9± 3.6	11.4± 3.9	11.1± 4.7	10.5± 4.3

Target and E/T ratio, see Table 5. The values are expressed as the mean ± S.D.

TABLE 7. COMPARISON OF NK ACTIVITY OF SPLEEN CELL FRACTIONS FROM 3LL TUMOR BEARING MICE AGAINST 3LL TARGET CELLS

Fraction	Days after tumor transplantation				
	0	3	5	10	15
Unfractionated	17.8± 3.7	14.2± 4.3	12.5± 3.8	8.4± 3.5	7.1± 3.5
Adherent	17.0± 4.2	13.2± 3.6	12.4± 3.7	10.2± 3.3	10.0± 3.6
Nonadherent	18.5± 4.2	16.6± 3.2	15.1± 4.0	13.3± 4.6	9.9± 3.8
Anti-Thy 1+C	12.8± 5.0	8.7± 4.3	7.0± 4.2	6.8± 4.4	5.2± 4.7

The target cells were 3LL cells. 1×10^4 ^{51}Cr-labeled 3LL cells were mixed with 1×10^6 fractionated spleen cells (E/T ratio of 100 : 1). The values are expressed as the mean ± S.D.

TABLE 8. THE EFFECT OF ANTI-Thy 1.2 PLUS C ON THE NK ACTIVITY OF SPLEEN CELLS FROM MH-134 OR 3LL TUMOR BEARING MICE AGAINST EACH KIND OF TARGET CELL

Target cell	% Reduction by treatment with anti-Thy 1 puls C[a]						
	Days after MH-134 tumor transplantation						
	0	3	5	7	9	10	14
Fibroblast	15.6± 3.7		27.5± 5.2		32.5± 4.3		20.6± 15.3
M-HeLa	9.4± 4.9	18.4± 7.8		27.4± 6.0		30.0± 7.7	22.3± 6.8
	Days after 3LL tumor transplantation						
	0	1	3	5	10	15	18
3LL	30.8± 5.7		47.6± 7.8	53.6± 3.5	48.9± 9.2	47.5± 6.4	
M-HeLa	11.2± 5.7	10.8± 9.2	24.8± 6.5	30.7± 4.9	37.4± 4.0	41.3± 5.6	14.6± 6.2

a Percent reduction in lysis was calculated as shown in Materials and Methods.

The effector to target ratio was 100 : 1 in all instances. The values are expressed as the mean ± S.D.

nonadherent cell fractions. When the nonadherent cell fraction was treated with anti-Thy 1.2 serum and complement, the activity decreased (Table 4, Fig. 3).

Likewise, the NK activity to M-HeLa target cells, decreased as the cancer progressed, showed about half of the normal activity on the 10th day, and nearly disappeared on the 17th day after tumor implantation. The removal of T cells

did not significantly affect the activity (Table 5).

NK activity of mice with 3LL cancer. The NK activity of spleen cells from mice with 3LL cancer to primary culture cells derived from 3LL and M-HeLa decreased with the progress of the cancer, as in the mice with MH-134 cancer. In the case of M-HeLa target cells, the nonadherent cell fraction showed a relatively high NK activity, and, by treatment with anti-Thy 1.2 serum and complement, the group with 3LL cancer showed a larger decrease in activity than the group without cancer (Table 6). When 3LL primary culture cells were used as the target cells (Table 7) in the group without cancer after removal of T cells less of a decrease in activity was noted than when M-HeLa or fetal lung fibroblasts were the target cells. The % reduction of NK activity through the removal of T cells became higher with the progress of cancer (Table 8).

DISCUSSION

Among the cells showing anti-tumor effects, attention has been paid recently to natural killer (NK) cells and K cells, which have antibody-dependent cellular cytotoxicity (ADCC).

In nude mice, which have not T cell function but high NK activity, the incidence of spontaneous cancer is low contrary to expectation (3, 4). In beige mutation mice (5, 6), genetically lacking NK activity, tumor resistance is low in spite of the intact anti-tumor activities of T cells and macrophages (7, 8). These facts show that the role of NK cells in resistance against tumors is significant. NK activity is reported to increase temporarily 15 h after inoculation of tumor cells (9), return to the normal level on the 3rd day and be suppressed after 5 days (10, 11). We also noted a decrease in NK activity with an increase in tumor size starting on the 5th day after tumor (MH-134) transplantation. By treatment with monoclonal anti-Thy 1.2 serum and complement, a significant decrease in activity was not seen in normal mice, but the activity decreased significantly in mice with 3LL cancer.

Herberman *et al.* (10) observed a decrease in ADCC when tumor cells were used as the target cells, while Becker and Kelin (11) found no change with red blood cells as the target cells, and Ghaffer *et al.* (12) reported increased activity with chicken red blood cells as the target cells. We observed increased activity in mice with MH-134 and 3LL cancers as well as spontaneous breast cancer. ADCC was increased by elimination of adherent cells, and after treatment with anti-Thy 1.2 serum and complement, ADCC decreased significantly to the level of spleen cells from mice without cancer. Therefore, the increase in ADCC in tumor bearing mice seems mainly attributable to T cells.

Santoni *et al.* (10, 13) studied the relation between ADCC effector cells (K cells) and NK cells in the mouse, and stated that K and NK cells might be the same, or at least be duplicates to a fairly large extent. If so, however, how can

the phenomenon of dissociation, *i.e.*, increased ADCC activity and decreased NK activity, observed in mice with cancer be explained? Herberman (14) proposed that the NK cells might be prethymic T cells and differentiate into T cells under the influence of thymus. With this hypothesis, the decrease in NK activity and increase in ADCC activity of spleen cells from mice with cancer may be explained by supposing that NK cells, distributed in the spleen, differentiate into T cells and lose NK activity, leaving K cell activity. However, another possibility is that NK activity and ADCC have different mechanisms of killing. Specifically, ADCC is markedly inhibited by such substances as Protein A and agglutinating IgG in combining specifically with the Fc portion of IgG, but NK activity is not influenced. Further, NK activity of the mouse decreases by treatment with trypsin or steroid, but ADCC activity is hardly affected. This indicates that NK activity does not have the same mechanism as ADCC. Therefore, there is a possibility that a certain humoral factor is responsible for the dissociation of NK activity and ADCC in cancer.

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