抗アシアロGM1血清処理ヌードマウスにおける牛白血病由来株化細胞(FLK)の増殖促進とNKおよびK細胞活性におよぼす血清の効果

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Enhancement of Growth of Bovine Leukemia Cell Line (FLK) by Anti-Asialo GM_1 Serum and the Effect of the Serum on NK and K Cell Activities in Nude Mice

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ABSTRACT. The enhancement of tumor growth in athymic nude mice transplanted with bovine leukemia cell line (FLK) was observed by inoculation of anti-asialo GM_1 (gangliotetraglycosylceramide) serum which has been shown to eliminate natural killer (NK) activity *in vitro* and *in vivo*. The biological mechanisms for the enhancement were investigated by examining NK activity and antibody-dependent cellular cytotoxicity (ADCC) of the spleen cells of the nude mice. FLK cells, that are weakly sensitive to NK cell-mediated immunolysis, were also killed by normal spleen cells of nude mice in the presence of specific antibodies to FLK cells. The ADCC was suppressed by inoculation of anti-asialo GM_1 serum, which indicates that ADCC-functioning killer (K) cells too have asialo GM_1 as NK cells. These results suggest that normal nude mice reject transplanted FLK cells by K cells as well as NK cells and inoculation of anti-asialo GM_1 serum allows the tumor growth through elimination of both the NK and K cell activities.

It is commonly conceived that the effector cells participating in cellular cytotoxic reaction are immune-killer T lymphocytes, killer (K) cells and macrophages. Recently, natural killer (NK) cells with non-T lymphocyte and non-macrophage nature have received widespread attention as effector cells [9, 10, 14, 26]. Since NK cells present in unprimed animals can lyse a veriety of target cells *in vitro*, they are considered to play a major role in immunosurveillance and protection agatinst virus infection and tumor growth.

Congenitally athymic nude mice are often used in studying NK cells since the nude mice have a higher NK activity than normal mice and the influences of T cell-mediated immune responses can be excluded [8]. The heterotransplantation of bovine lymphosarcoma and bovine leukemia cell line was successful in the nude mice [22–25]. Immunological reactions of the host for the heterotransplantation of bovine leukemia cell line (FLK) are studied in the present paper. NK cells can be eliminated by the treatment of anti-asialo GM_1 serum *in vitro* and *in vivo* [5, 13]. In the present experiment, it was observed that injection of anti-asialo GM_1 serum into nude mice enhanced the growth of the transplanted FLK cells. The enhanced mechanism of the tumor growth was studied by examining NK and K cell activities. This study will provide further information on the relationship of NK and K cells to the host defence mechanisms in nude mice.

MATERIALS AND METHODS

Nude mice: The nude mice used in this study were syngeneic mice of BALB/c strain provided by the Institute of Medical Science, University of Tokyo, Tokyo,

Japan. They were bred under specific pathogen-free conditions.

Cells: Fetal lamb kidney (FLK) cell line, persistently infected with bovine leukemia virus (BLV) was kindly provided by Dr. Van Der Maaten, NADC, Ames, Iowa. The cells were grown in Eagle's minimum essential medium (Nissui, Tokyo, Japan) containing 10% fetal calf serum (FCS) at 37°C in a humid atmosphere with 5% CO_2. YAC-1 cells, derived from Moloney virus-induced lymphoma in A/Sn mice, were kindly provided by Dr. T. Kamiyama, National Insitute of Health, Tokyo, Japan. The cells were maintained *in vitro* in RPMI 1640 (Nissui, Tokyo, Japan) plus 10% FCS.

Preparation of effector cells: Spleen cell suspensions were prepared by mincing the spleens with scissors. Cells were passed through 200-gauge stainless-steel mesh and washed once by centrifugation. The cells were adjusted to 1.0×10^7 cells/m*l* in RPMI 1640 containing 10% FCS.

Cytotoxicity assay: [^{75}Se] selenomethionine release assay was performed. The technique used was essentially similar to that of Brooks [1] and Brooks *et al.* [2]. Target cells were cultured for 19 to 24 hours at 37°C in medium containing [^{75}Se] selenomethionine (5 μCi/m*l* medium) (specific activity 0.6 to 4 Ci/m mol, The Radiochemical Centre, Amersham, England). The cells were washed four times by centrifugation and resuspended in RPMI 1640 at a concentration of 1.0×10^5 cells/m*l*. Isotope-labelled target cells (0.1 m*l*) and the effector cells (0.1 m*l*) were each placed in duplicate or triplicate in a well of round-bottomed microplate (Nunc, Roskilde, Denmark) at an E/T ratio of 100. The cells were centrifuged for 5 min at 400 g and incubated for 20 hours at 37°C in humid atmosphere with 5% CO_2. After incubation the supernatant fluids were collected using Titertek Harvesting Flames (Flow Lab., Mclean, Virg.) and counted for radioactivity in a gamma scintillation counter (Auto Well Gamma System ARC-501, Aloka, Tokyo, Japan). The cytotoxic effect of spleen cells was calculated by the following formula:

$$\text{Percent of specific lysis} = \frac{\text{test release cpm} - \text{spontaneous release cpm}}{\text{maximum release cpm} - \text{spontaneous release cpm}} \times 100$$

Maximum release was determined from the degradation of labelled target cells after addition of 0.1 m*l* of 0.1% sodium dodecylsulfate.

Antibody-dependent cellular cytotoxicity (ADCC) test: [^{75}Se] selenomethionine-labelled target cell suspension was added to various concentrations of heat-inactivated anti-FLK mouse serum or normal mouse serum and incubated for 45 min at 37°C. Antibody-sensitized target cells were washed 3 times and adjusted to a concentration of 1.0×10^5 cells/m*l*. ADCC assays were performed in the same manner as described in the cytotoxicity assay.

Anti-FLK serum: Anti-FLK sera were prepared in heterozygous and nude mice. FLK cells (2.0×10^7 cells) fixed by glutaraldehyde were inoculated subcutaneously into a mouse with Freund's complete adjuvant. The mouse was intraperitoneally boostered 4 and 5 weeks after the first injection with the same dose of cells without adjuvant. The serum was collected 3 days after the last injection. The serum titer was 1:640, when examined by complement-dependent antibody cytotoxicity (CDAC) test [25]. Another anti-FLK nude mouse serum was prepared by subcutaneous inoculation of glutaraldehyde-fixed

cells (1.5×10^7 cells) with Freund's complete adjuvant. As a booster, the cells (7.0×10^6 cells) were inoculated without adjuvant 2 weeks later. Four days after the second inoculation, the mouse was inoculated with 2.0×10^6 viable FLK cells. Serum titer collected from the mouse 28 days after the last inoculation was 1:8 by CDAC test.

Anti-asialo GM_1 serum: Asialo GM_1 was prepared from purified bovine brain GM_1 ganglioside as described in the previous paper [20]. Each footpad and multiple numbers of intracutaneous sites of rabbits were injected with 2 m*l* of an emulsion containing 1 mg asialo GM_1 and 1 mg methylated bovine serum albumin (Me-BSA) and 1 m*l* Freund's complete adjuvant. Four weeks after the first inoculation, each rabbit was intramuscularly boostered with the same amount of asialo GM_1 and Me-BSA in Freund's incomplete adjuvant. The rabbit antisera were harvested 2 weeks after the second injection. After the antibodies to Me-BSA was removed by an affinity chromatography, the specificity of the antiserum was examined by a semiquantitative complement fixation test as previously described [20]. The titer of the antiserum used in this experiment was 1:640 against asialo GM_1 but the serum did not cross-react with gangliosides GM_1 and GD_{1b} at 1:10 dilution. Asialo GM_1 was demonstrated on cell surface of spleen cells from nude mice but not on that of FLK cells by indirect fluorescent antibody (FA) technique.

In vitro treatment of nude mouse spleen cells with anti-asialo GM_1 serum: Spleen cells of nude mouse were treated with anti-asialo GM_1 serum and rabbit complement. Anti-asialo GM_1 serum (0.1 m*l*) and rabbit serum (0.2 m*l*) were mixed with spleen cell suspension (0.7 m*l*) and incubated for 45 min at 37°C. The rabbit serum which is not cytotoxic for nude mouse spleen cells was used as complement. After washing the spleen cells, NK and ADCC reactivities were examined.

Inoculation of FLK cells and anti-asialo GM_1 serum to nude mice: Since FLK cells are shown to be transplantable in nude mice [24], the cells passaged in nude mice or maintained *in vitro* were inoculated subcutaneously on the back with different numbers of cells. In Exp. 1, a small number of FLK cells (1.0×10^5 cells/0.1 m*l*) collected from a tumor developed in nude mouse were inoculated into animals. In Exp. 2, 1.2×10^6 cells cultured *in vitro* were inoculated. One group of mice in each experiment was injected subcutaneously with 0.1 m*l* of anti-asialo GM_1 serum. The antiserum treatment was repeated 4 times every 5 to 7 days. The tumor growth in the nude mice was examined every day.

Statistical analysis: Data were analysed statistically by the Student's t-test and χ^2 test.

Results

In vivo effect of anti-asialo GM_1 serum on the development of FLK tumors—To examine the effect of anti-asialo GM_1 serum *in vivo*, FLK inoculated nude mice were injected with the antiserum and observed for tumor development. FLK cells were inoculated subcutaneously to twelve 9-weeks-old and ten 7-weeks-old nude mice in two separate experiments. Half of the mice were inoculated subcutaneously with anti-asialo GM_1 serum. Table 1 shows the tumor incidence in the mice treated with anti-asialo GM_1 serum. Tumor growth was observed in 5/6 (Exp. 1) and 5/5 (Exp. 2) of the antiserum-treated mice whereas no tumor appeared in the control groups during the observation period ($p<0.01$, χ^2 test). Antibody titers against FLK cells were estimated by

Table 1. Effect of anti-asialo GM_1 serum on the development of tumors in BALB/c nude mice

Exp.	No. FLK cells inoculated	Age (weeks)	Inoculation of anti-asialo GM_1 serum	Tumor incidence	Median days of tumor appeared	CDAC titer[a]	
						20[b]	37
1	1.0×10^5	9	+	5/6[c]	15.3[c] (9, 12, 12, 16, 19, >37)[e]	2, 2, 4	4, 8, 8
			−	0/6	−(>20, >20, >20, >37, >37, >37)	2, 4, 32	16, 16, 32
2	1.2×10^6	7	+	5/5[c]	7.9[d] (5, 7, 10, 10, 11)	NT[f]	NT
			−	0/5	−(>13, >13, >15, >15, >15)	NT	NT

a) Antibodies against FLK cells were examined by CDAC test.
b) Days after FLK inoculation.
c) $p<0.01$, (χ^2 test).
d) $p<0.001$, (t-test).
e) Days after inoculation.
f) Not tested.

CDAC test [25] 20 and 37 days after FLK inoculation in Exp. 1. Increase of antibody titer was observed in both groups; the antibody titer being higher in non-treated group than the antiserum-treated group. No CDAC antibody in FLK-uninoculated control nude mice were detected in serum before and after FLK inoculation (<2).

NK and ADCC reactivities of nude mice and the effect of anti-asialo GM_1 serum—The effect of anti-asialo GM_1 serum on NK and ADCC reactivities in nude mice was examined in *in vitro* and *in vivo*. Spleen cells of three 6-week-old nude mice were treated with anti-asialo GM_1 serum and complement *in vitro* (Table 2). FLK cells used for target cells in ADCC assay were treated with anti-FLK heterozygous mouse serum (titer; 1:640 by CDAC test) diluted 1000 times with medium. NK activity and ADCC were expressed as cytotoxicity of spleen cells against untreated and antibody-coated FLK target cells, respectively. The result shows that treatment of spleen cells with anti-asialo GM_1 serum and complement decreased not only NK activity of spleen cells against both FLK and YAC-1 cells ($p<0.01$, Student's t-test) but also ADCC activity against FLK cells ($p<0.001$) when compared to the normal control.

Table 3 shows the *in vivo* effect of anti-asialo GM_1 serum on NK and ADCC reactivities in nude mice. A total of twelve 11-weeks-old nude mice was divided into four groups. One group of mice were inoculated with FLK cells (1.1×10^7/0.1 m*l*) and anti-asialo GM_1 serum. The other two groups were inoculated with either FLK cells or the antiserum, and the last group served as normal controls. Anti-asialo GM_1 serum was repeatedly injected on the day before the experiment. The test was done one week after FLK inoculation. The result shows that FLK cells, which were treated with anti-FLK mouse serum, were killed by spleen cells of normal or FLK inoculated nude mice (ADCC) but the untreated FLK cells showed no sensitivity against lysis by the spleen cells of the four groups of nude mice (NK). ADCC against FLK cells was suppressed significantly in both groups of mice treated with anti-asialo GM_1 serum and anti-asialo GM_1 serum plus FLK cells ($p<0.05$, Student's t-test) when compared to normal nude mice. NK activity against YAC-1 cells was significantly suppressed in groups treated with anti-asialo GM_1 serum ($p<0.01$).

ADCC activity of nude mice against

Table 2. *In vitro* effect of anti-asialo GM_1 serum on NK and ADCC reactivities of spleen cells from BALB/c nude mice

Treatment of spleen cells	Percent specific lysis (mean% ±SE) of		
	FLK cells		YAC-1 cells
	NK activity	ADCC[a]	NK activity
Normal control	7.1±1.9	16.5±2.1[b]	31.0±4.2
Anti-asialo GM_1 serum+complement[c]	−3.9±0.6[d]	−4.1±0.5[e]	−1.9±1.4[d]

a) Total cytotoxicity against antibody-coated FLK target cells.
b) $p<0.05$ (t-test) when compared to the NK activity against FLK cells.
c) Spleen cells were incubated with anti-asialo GM_1 serum and rabbit complement for 45 min at 37°C.
d) $p<0.01$ (t-test) when compared to normal control.
e) $p<0.001$ (t-test) when compared to normal control.

Table 3. NK and ADCC reactivities of spleen cells from BALB/c nude mice inoculated with FLK cells and anti-asialo GM_1 serum

Treatment	Percent specific lysis (mean% ±SE) of		
	FLK cells		YAC-1 cells
	NK activity[a]	ADCC[b]	NK activity
Normal control	−3.4±0.5	14.8±2.9[c]	15.2±2.7
Anti-asialo GM_1 serum[d]	−4.6±0.2	3.7±2.1[e, f]	1.2±1.7[g]
FLK cells[h]	−3.2±0.8	11.4±3.3[c]	9.2±2.1[e]
FLK cells+anti-asialo GM_1 serum	−5.4±0.4	3.8±2.6[e, i]	0.6±1.8[g]

a) Total cytotoxicity against antibody-uncoated FLK target cells.
b) Total cytotoxicity against antibody-coated FLK target cells.
c) $p<0.01$ (t-test) when compared to the NK activity of the same spleen cell preparation against antibody-uncoated FLK target cells.
d) Anti-asialo GM_1 serum was inoculated subcutaneously 7 days and 18 hours before experiment.
e) $p<0.02$ (t-test) when compared to the NK activity of the same spleen cell preparation against antibody-uncoated FLK target cells.
f) $p<0.05$ (t-test) when compared to normal control.
g) $p<0.01$ (t-test) when compared to normal control.
h) Nude mice were inoculated with FLK cells (1.1×10^7) 7 days before experiment.
i) $p<0.05$ (t-test) when compared to the NK activity of the same spleen cell preparation against antibody-uncoated FLK target cells.

FLK cells sensitized with anti-FLK serum from heterozygous or nude mouse—ADCC against FLK cells was examined using spleen cells from three 11-weeks-old nude mice (Table 4). Isotope-labelled FLK cells were incubated with various concentrations of normal or anti-FLK serum from heterozygous mouse (CDAC titer; 1:640). The result showed that FLK cells treated with anti-FLK serum were killed by spleen cells at serum concentrations of 1:100 to 1:10000. The sensitivity of target cells treated with normal serum to spleen cells of nude mouse was almost at the same level as that of non-treated target cells.

ADCC mediating activity of the antibody raised against FLK cells in nude mouse was examined on spleen cells from 8-weeks-old nude mouse (Table 4).

Table 4. ADCC activity of nude mouse spleen cells against FLK cells treated with anti-FLK serum of heterozygous or nude mouse[a)]

Serum dilution	Percent specific lysis			
	Heterozygous mouse serum		Nude mouse serum	
	Immune serum[a)]	Normal serum	Immune serum[c)]	Normal serum
1:10	9.9±0.3[d)]	6.7±0.6	ND[e)]	ND
1:100	20.0±1.2	11.7±1.2	10.3	0.2
1:1000	28.0±2.0	10.9±2.2	4.9	1.0
1:10000	28.1±1.2	12.5±0.5	8.2	−0.9
1:100000	ND	ND	2.2	−1.8
Without serum	7.6±1.3	7.6±1.3	0.6	0.6

a) FLK target cells were incubated with anti-FLK or normal serum for 45 min at 37°C and then incubated with effector cells at E/T ratio of 100.
b) CDAC titer; 1:640.
c) CDAC titer; 1:8.
d) Mean%±SE of spleen cell preparations from 3 mice.
e) Not done.

Titer of the serum against FLK cells was 1:8 when examined by CDAC test. FLK target cells were incubated with various dilutions of normal or anti-FLK serum of nude mouse. The result shows that FLK cells treated with anti-FLK serum were killed by spleen cells at the serum concentrations of 1:100 to 1:10000. Untreated FLK cells and FLK cells treated with normal nude mouse sera were not killed by the spleen cells from the same nude mouse at all. However, the degree of ADCC activity with anti-FLK nude mouse serum was weaker than that with anti-FLK heterozygous mouse serum (Table 4).

Discussion

Biological and immunological responses of nude mice against heterotransplantation of tumor cells and line cells from bovine leukemia have been investigated [15, 22, 23, 25]. NK cells in nude mice are thought to be an important effector cells in rejecting syngeneic, allogeneic and xenogeneic tumors [8]. A surface marker of NK cells of mouse could be detected with antiserum against mouse brain tissue (anti-brain-associated θ antigen) [6] and antibody to asialo GM_1 in the serum was responsible for the anti-NK activity [4, 12, 30]. Habu *et al.* [5] and Kasai *et al.* [13] showed that suppression of NK activity of nude mice by injection of anti-asialo GM_1 serum resulted in the enhancement of the tumor incidence and growth of YAC-1 and/or RL♂1 cells. Because these tumor cells are highly sensitive to NK cells, the enchancement of the tumor incidence appear to be due to inactivation of NK cell activity by the serum. The time course study using ^{75}Se labelled YAC-1 cells in the preliminary experiments revealed that cytotoxic reactions occurred at 4 hours of incubation and the activity reached to the maximum at 20 hours. Since the activities at 4 and 20 hours were suppressed after treatment of nude mice with anti-asialo GM_1 serum, the activity observed by ^{75}Se release assay appeared to be NK cell activity.

In the present study, nude mice inoculated with anti-asialo GM_1 serum developed trasplanted FLK tumors with high incidence. These results made us expect

that the suppression of NK activity by anti-asialo GM_1 serum facilitated FLK tumor development. However, it was found that FLK cells were not highly susceptible for killing by NK cells of nude mice. It is probable that FLK cells do not possess asialo GM_1 on the surface of the cells since anti-asialo GM_1 serum does not react to FLK cells when examined by FA test (data are not shown). Further, FLK tumor might not develop in nude mice if the antiserum exerts direct cytotoxic activity against FLK cells. Therefore, it seems that the effect of anti-asialo GM_1 serum observed in the present study is not directed to the FLK cells themselves. The macrophages do not seem to play a major role in rejection of FLK tumor since the cytotoxic activity of peritoneal exudate cells against YAC-1 cells is thoroughly retained after inoculation with anti-asialo GM_1 serum to nude mice but FLK cells are not killed at all by peritoneal exudate cells or splenic adherent cells of these nude mice [15]. Therefore, another immune mechanism was supposed to play a role in rejection of FLK cells.

The K cells participating in ADCC is known as another important effector cell for elimination of tumor cells. Like NK cells, active cells for ADCC belong to non-T and non-B lymphocytes and are demonstrated in the spleen cells of nude mice [27] though they have Fc receptors as B lymphocytes [3, 17, 29]. Though the relationship of NK cells to K cells is still unknown, recent studies indicated that NK cells can also function as K cells against certain cells [7, 11, 16, 21, 27]. ADCC reactivity of the spleen cells from nude mice, therefore, was subsequently examined in this study. The results indicate that effector cells in spleens of nude mice kill the transplanted FLK cells with a specific antibody and that the ADCC-active cells were suppressed in *in vivo* and *in vitro* by treatment with anti-asialo GM_1 serum (Tables 2 and 3). The nude mice immunized with FLK cells (Table 4) or inoculated with a small number of FLK cells [15] developed antibody against FLK cells as demonstrated by CDAC and ADCC tests. ADCC may be an effective defence mechanism of the host since ADCC can be exhibited even in the presence of low amount of antibody. Since ADCC reactivities detected in the present study are relatively low, it is difficult to draw any conclusions that nude mice eliminate FLK cells only by ADCC mechanism. In the present study, some nude mice showed weak NK activity against FLK cells (Tables 2 and 4). The activity was noted to be age dependent (unpublished data). Though the possibility that young nude mice reject transplanted FLK cells by NK cells can not be excluded, the role of K cells can be supposed as one of the candidate for effector cells in rejecting FLK cells from the body.

The present paper is the first declaration that ADCC-functioning K cells too have asialo GM_1 as NK cells. The lymphocytes with asialo GM_1 were detected in a large number (about 50%) of normal mouse spleen cells but they were detected in only a few percentage (about 5%) of nude mouse spleen cells [28]. The present results suggest that a low percentage of asialo GM_1-positive cells in the nude mouse showed both ADCC and NK activities. However, these results alone will not clarify whether the cells showing ADCC activity to FLK cells are different from the cells showing NK activity to YAC-1 cells. In normal mice, asialo GM_1 is not a specific marker of NK and/or K cells. Suppressor T lymphocyte was also eliminated by the treatment of anti-asialo GM_1 serum *in vitro* [19]. In normal rats,

macrophages were also eliminated by injection of anti-asialo GM_1 serum [18]. Therefore, the enhancing mechanism of heterotrasplantation of tumor by injection of anti-asialo GM_1 serum should be studied in more details in the future.

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要　約

抗アシアロ GM_1 血清処理ヌードマウスにおける牛白血病由来株化細胞 (FLK) の増殖促進と NK および K 細胞活性におよぼす血清の効果: 児玉　洋・小沼　操・山本慎一・見上　彪・伊沢久夫・松本耕三[1)]・内貴正治[2)] (北海道大学獣医学部家畜伝染病学講座・[2)]家畜生化学講座，[1)]北海道大学医学部附属実験動物施設)——ナチュラルキラー(NK)活性を *in vitro* および *in vivo* で抑制する抗アシアロ GM_1 (gangliotetraglycosylceramide) 血清をヌードマウスに接種すると移植された牛白血病由来株化細胞 (FLK) の増殖が促進された．腫瘍増殖促進機序を明らかにするため，ヌードマウス脾細胞の NK 活性および抗体依存細胞性細胞障害 (ADCC) を調べた．FLK 細胞は NK 細胞に弱い感受性を示すが，抗 FLK 細胞抗体存在下で正常ヌードマウス脾細胞に強い感受性を示した．ADCC は抗アシアロ GM_1 血清接種により抑制され，ADCC に関与するキラー (K) 細胞も，NK 細胞同様アシアロ GM_1 を持つことが示唆された．これらの結果は，ヌードマウスは移植された FLK 細胞を NK 細胞同様K細胞によっても排除することを示唆しており，抗アシアロ GM_1 血清接種により NK および K 細胞活性が抑制されたため腫瘍が増殖したものと考えられる．