Impairment of K Cell Activity by Inoculation of Bovine Leukemia Cell Line (FLK) in Nude Mice

Shin-ichi YAMAMOTO, Misao ONUMA, Hiroshi KODAMA, Takeshi MIKAMI,
Hisao IZAWA, and Kozo MATSUMOTO¹⁾

Department of Epizootiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, and

¹⁾Institute for Animal Experiment, School of Medicine, Hokkaido University, Sapporo 060

(Received 15 November 1982/Accepted 3 June 1983)

ABSTRACT. In athymic nude mice previously inoculated with bovine leukemia cell line (fetal lamb kidney; FLK) the clearance of the same cells was markedly delayed. To know the mechanisms of impairment of clearance rate, we examined natural killer (NK) and killer (K) cell activities of spleen cells against FLK cells by 8 hr-⁵¹Cr release assay. Splenic K cell activity against FLK cells markedly decreased in nude mice treated previously with viable FLK cells when compared to the activity of spleen cells from untreated nude mice. However, there were no significant differences in NK cell activity against FLK cells and YAC-1 cells between the two groups of mice. Treatment of nude mice with glutaraldehyde-fixed and killed FLK cells had no influence on both NK and K cell activities. These results suggest that inoculation of viable FLK cells impairs K cell activity of nude mice and therefore the elimination of FLK cells from the body was delayed. The impairment of K cell activity may result in progress of tumors in nude mice.

Bovine leukemia virus (BLV) is the causative agent of bovine lymphosarcoma [9]. Recent serologic surveys have shown that BLV infection is widely spread among cattle [6, 10], while the occurrence of leukemia is low. This evidence suggests that an immunosurveillance system in host may play a role in the inhibition of the progress of the disease [13, 16]. Humoral and cellular immune responses against BLV or bovine leukemia cell lines have been investigated in cattle and sheep [2, 16], and the mitogenic responses of lymphocytes from BLV-infected animals have been investigated [17]. Not only T-cell and B-cell immunity but also natural killer (NK) cell, killer (K) cell and macrophage have been recognized as important mechanisms in immunosurveillance system [3–5]. The role of cellular immunity in immunosurveillance for elimination of

tumor cells is as yet unknown in bovine leukemia system. Fetal lamb kidney (FLK) cell line persistently infected with BLV has been shown to have tumorigenicity in athymic nude mice [11, 12], and host immune mechanisms against FLK cells have already been investigated [8]. The purpose of the present study is to investigate how heterotransplantable FLK cells are eliminated by host immune responses in nude mice.

MATERIALS AND METHODS

Nude mice: Syngeneic nude mice of BALB/c strain were provided by the Institute of Medical Science, University of Tokyo, Tokyo, Japan. They were bred under specific pathogen-free conditions. **Cells:** FLK cell line was kindly provided by Dr. Van Der Maaten, NADC, Ames, Iowa. YAC-1 cell line, derived from a Moloney virus-induced lymphoma in A/

Sn mice, was kindly provided by Dr. T. Kamiyama, National Institute of Health, Tokyo, Japan. Cultivation of the line cells and culture medium were described in the previous paper [12].

Effector cells: Spleen cell suspensions were prepared by mincing the spleens with scissors. Cells were passed through 200-gauge stainless-steel mesh, washed once by centrifugation and then adjusted to 1.0 to 10×10^6 cells/ml in RPMI 1640 (Nissui, Tokyo, Japan) (pH 7.4) containing 10% fetal calf serum (FCS).

Radioisotope labeling of cells: FLK or YAC-1 cells were washed once in medium and resuspended in $50 \mu\text{Ci}$ of $\text{Na}_2^{51}\text{CrO}_4$ solution (specific activity 250 to $500 \mu\text{Ci}/\text{mg}$ Cr, The Radiochemical Centre, Amersham, England). Cell suspension was incubated for one hour at 37°C , and then washed four times. The cells were resuspended in RPMI 1640 containing 10% FCS at a concentration of 1.0×10^5 cells/ml.

^{75}Se selenomethionine-labeled FLK cells were used for the experiment on *in vivo* clearance of the cells. FLK cells cultured for 24 hr at 37°C in medium containing ^{75}Se selenomethionine ($5 \mu\text{Ci}/\text{ml}$ medium) (specific activity 0.6 to 4 Ci/mMol, The Radiochemical Centre, Amersham, England) were washed four times and inoculated subcutaneously on the back of nude mice.

NK assay: Isotope-labeled target cells (0.1 ml) and the effector cells (0.1 ml) were each placed in duplicate or triplicate in a well of round-bottomed microplate (Nunc, Roskilde, Denmark) at an effector-target (E/T) cell ratio of 10 to 100. The cells were centrifuged for 5 min at 400 g and incubated for 8 hr at 37°C in a humid atmosphere with 5% CO_2 . After incubation the supernatant fluids were collected using Titertek Harvesting Flasks (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:

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

Maximum release was determined for labeled target cells degraded by the addition of 0.1 ml of 0.1% sodium dodecyl-sulfate.

Antibody-dependent cellular cytotoxicity (ADCC) assay: ^{51}Cr -labeled target FLK cell suspension was added to heat-inactivated anti-FLK mouse serum (1:1000 dilution). After incubation for 45 min at 37°C , antibody-sensitized target cells were washed three times and adjusted to a concentration of 1.0×10^5 cells/ml. ADCC assays were performed in the same manner as described in the NK assay. Percentage of lysis for ADCC was determined by subtraction of % lysis of NK cell activity from total cytotoxicity against antibody-coated cells.

Anti-FLK mouse serum: Anti-FLK serum was prepared in heterozygous mouse. FLK cells (2×10^7 cells) fixed by 0.6% glutaraldehyde were inoculated subcutaneously into mouse with complete Freund's adjuvant. The mouse was intraperitoneally boosted 4 and 5 weeks after the first injection with the same amount of cells without adjuvant. The serum was collected 3 days after the last injection. The serum titer was 1:1280 when examined by complement-dependent antibody cytotoxicity (CDAC) test [7].

CDAC test: Antibodies against FLK cells in nude mice were estimated by CDAC test as described previously [7].

In vivo clearance of FLK cells: Eight-

weeks-old nude mice were inoculated subcutaneously on the back with FLK cells (1.2×10^6 cells) and another group of the same age served as controls. Twelve days later, both groups of mice were challenged subcutaneously with ^{75}Se -labeled FLK cells (2.9×10^5 cells) in the opposite side on the back. Radioactivity associated with the liver, spleen, kidney, lung and inoculated site was determined 18 and 66 hr after inoculation.

Treatment of nude mice killed or live cells: Eight-weeks-old nude mice were divided into two groups. One group was inoculated intraperitoneally with 1.0×10^7 fixed FLK cells weekly with glutaraldehyde for three times, and then inoculated with cells (1.0×10^7 cells) homogenized with Dounce type homogenizer one week later. Second group of mice were once inoculated subcutaneously with live FLK cells (2×10^6 cells). In each group, 3 mice without inoculation served as control. NK and K cell activities of these mice were examined 30 or 32 days after inoculation of FLK cells. The level of antibodies against FLK cells was also estimated by CDAC test at the same time. Three nude mice from 2 groups and control were used in each test.

Statistical analysis: Data were analyzed statistically by the Student's t-test.

RESULTS

In vivo clearance of FLK cells: To know the *in vivo* clearance of FLK cells in nude mice, mice treated with viable FLK cells and untreated control mice were inoculated with ^{75}Se -labeled viable FLK cells. Internal organs and the inoculation site of the labeled FLK cells were collected from 2 nude mice in each group 18 and 66 hr after inoculation and were counted for radioactivity (Table 1). The results showed that 52.5% and 27.1% of radioactivity remained in the inoculated site of ^{75}Se -labeled FLK cells in mice treated with viable FLK cells 18 and 66 hr after inoculation, respectively. However, only 11.4% and 6.6% of radioactivity was detected in control mice 18 and 66 hr after inoculation, respectively. Radioactivity remained in the liver, kidney, spleen and lung in mice treated with viable FLK cells 66 hr after inoculation at higher level than that of control mice. This result suggests that the elimination of FLK cells from the inoculated site is reduced if mice are treated with viable FLK cells before challenge.

NK and K cell activities of nude mice against FLK and YAC-1 cells: It was interesting to know what mechanisms play for the elimination of FLK cells from nude mice.

Table 1. Clearance of radiolabeled FLK cells inoculated subcutaneously in BALB/c nude mice

Pretreatment	Hours after challenge with radiolabeled FLK cells ¹⁾	Percentage of radioactivity remained				
		Inoculated site	liver	kidney	spleen	lung
FLK cells ²⁾	18	52.5 ³⁾ (19.1) ⁴⁾	4.53 (1.45)	1.45 (0.48)	0.162 (0.038)	0.181 (0.105)
	66	27.1 (8.3)	11.73 (2.24)	3.43 (0.84)	0.393 (0.092)	0.570 (0.137)
Control	18	11.4 (4.2)	5.05 (1.13)	1.56 (0.30)	0.167 (0.025)	0.194 (0.045)
	66	6.6 (7.9)	5.10 (3.06)	1.64 (1.18)	0.218 (0.117)	0.206 (0.143)

1) Total radioactivity was 488,737 cpm/ 2.9×10^5 FLK cells.

2) FLK cells (1.2×10^6 viable cells) were inoculated subcutaneously 12 days before challenge with radiolabeled FLK cells.

3) Mean percentage from 2 nude mice.

4) SD of the mean.

To clarify this point, sensitivity of FLK cells to NK or K cell-mediated lysis were examined (Fig. 1). Spleen cells of three 13-weeks-old nude mice were tested for NK and K cell activities against FLK cells with effector/target (E/T) cell ratios of 10, 33 and 100. YAC-1 cells were used as control target cells of NK assay. Since the highest cytotoxicity by anti-FLK serum (ADCC titer of 20.8%) and the minimal cytotoxicity by normal serum (ADCC titer of 2.5%) were observed against FLK cells at serum dilution of 1:1000 in the preliminary experiment, the dilution of 1:1000 was employed for evaluating K cell activity. Figure 1 shows that FLK cells treated with anti-FLK mouse serum are sensitive to K cell-mediated lysis. The highest cytotoxic reaction was observed at E/T cell ratio of 100. Further, it was demonstrated that FLK cells were also sensitive to NK cell-mediated lysis but the sensitivity of FLK cells was not so high as YAC-1 cells. These results suggest that these effector cells may play a role in elimination of FLK cells from the body.

Effect of treatment with live or killed FLK cells on NK and K cell activities of nude mice: Next experiment was conducted to know

Fig. 1. NK and K cell activities of nude mice against FLK and YAC-1 cells. Thirteen-weeks-old nude mice were used. Each circle and vertical line represent mean value and SE of 3 nude mice. Symbols represent the cytotoxicity of spleen cells against FLK cells or YAC-1 cells (○—○; NK cell activity) and FLK cells treated with anti-FLK mouse serum (●—●; K cell activity plus NK cell activity). E/T: Ratio of effector cells (E) to target cells (T).

Table 2. Effect of inoculation with live or killed FLK cells on NK and K cell activities

Exp. No.	Treatment ¹⁾	ADCC activity ²⁾	NK activity		CDAC titer
		FLK ³⁾	FLK	YAC-1 ³⁾	FLK
1 ⁴⁾	Killed FLK cells	14.2±1.7 ⁵⁾	16.3±1.3	20.4±3.7	2, 2, <2
	Non-treated	14.3±1.9	20.4±2.3	25.6±2.9	<2, <2, <2
2 ⁶⁾	Live FLK cells	1.5±1.3 ⁷⁾	13.3±2.1	28.9±2.5	16, 8, 8
	Non-treated	12.3±2.8	6.2±1.5	29.9±2.3	<2, <2, <2

1) Methods of treatment are indicated in the text.

2) ADCC activity was determined by subtraction of % lysis of NK cell activity from total cytotoxicity.

3) Target cells.

4) Tested at 32 days after inoculation.

5) Mean percentage ±SE of three nude mice.

6) Tested at 30 days after inoculation.

7) $p < 0.05$ when compared to the normal control.

the effect of inoculation with killed or live FLK cells on NK and K cell activities of nude mice. Thirty to 32 days after inoculation of the cells, NK cell activity against FLK and YAC-1 cells and K cell activity against FLK cells were examined to know whether suppression of NK or K cell activities occur because of the inoculation. Compared to NK and K cell activities of control mice, nude mice inoculated with killed FLK cells did not exert any influence on the activities (Table 2). However, K cell activity but not NK cell activity in mice inoculated with live FLK cells was markedly suppressed when compared to that of control mice ($p < 0.05$). Increase of antibody titer against FLK cells was observed in all of the mice inoculated with live cells.

DISCUSSION

The observation of *in vivo* clearance of isotope-labeled FLK cells from nude mice in the present study showed that treatment of viable FLK cells impaired clearance of the labeled FLK cells from the inoculated site. It was also found that suppression of ADCC activity of nude mice was occurred when the mice were treated with viable FLK cells but not by treatment with killed FLK cells. The radioactivity detected in the inoculated site presumably originated from viable FLK cells and cell components degraded by host cells. Detection of higher radioactivity in the inoculated site of mice treated with viable FLK cells than control mice (Table 1) indicated that suppression of the killing of FLK cells and removal of cell debris from the inoculated site occurred. Since FLK tumors are formed only in the inoculated site and not metastasized into internal organs (unpublished), the radioactivity associated with internal organs may be derived from cell components degraded by host cells. Remains of

tumor cells in the inoculated site for long period, even though considerable numbers of FLK cells are killed by host cells, offer good chances for development of FLK tumors in nude mice.

To examine the mechanisms of the impairment of tumor cell elimination, we tested NK and K cell activities of nude mice treated with FLK cells (Table 2). NK cell activity against FLK and YAC-1 cells was not affected by treatment with killed or live FLK cells. However, K cell activity was suppressed significantly by treatment with viable FLK cells. The results indicated that impairment of K cell activity occurred only when nude mice were inoculated with viable FLK cells but not with killed FLK cells. Though we used anti-FLK heterozygous mouse serum for evaluating K cell activity of spleen cells in the present study, it was demonstrated that nude mouse sera against FLK cells exert ADCC combined with spleen cells of normal nude mice [8]. The macrophage does not seem to play a major role for rejection of FLK tumors in nude mice since FLK cells are not killed at all by peritoneal exudate cells or splenic adherent cells [8]. Therefore, inoculation of viable FLK cells to nude mice causes the suppression of K cell activity and the impairment of elimination of tumor cells. This suppression of K cell activity may result in the progress of tumors. Similar observation was found in bovine and sheep systems. Peripheral blood lymphocytes from normal bovine and sheep showed natural cytotoxic activity against FLK cells [14, 18]. This cytotoxic activity was suppressed when animals were leukemic. Therefore, K and/or NK cells are thought to be important effector cells in rejecting tumor cells in rejecting tumor cells in bovine as well as nude mice.

Though the relationship of NK cells to

K cells is not well known, it is interesting to know if there are some correlations between the activities of these effector cells and *in vivo* tumor development or regression. Becker and Klein [1] demonstrated that tumor bearing mice have decreased NK cell activity, whereas ADCC directed against antibody-coated chicken red blood cells was not impaired. Santoni *et al.* [15] reported that nude mice bearing murine sarcoma virus-induced sarcoma had decreased not only NK cell but also ADCC activity. Thus the characteristics of the activity and the nature of the effector cells vary considerably among studies and they appear to depend on several parameters (target cell system and type of cytotoxicity assay). The mechanism of suppression of K cell activity by inoculation of viable FLK cells should be studied in the future.

ACKNOWLEDGEMENT. We wish to express our appreciation to Miss S. Tateshita for preparation of manuscript. This work was supported in part by a grant from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

1. Becker, S., and Klein, E. (1976). Decreased "natural killer" effect in tumor-bearing mice and its relation to the immunity against oncornavirus-determined cell surface antigens. *Eur. J. Immunol.* **6**, 892-898.
2. Bex, F., Bruck, C., Mammerickx, M., Portetle, D., Ghysdael, J., Cleuter, Y., Leclercq, M., Dekegel, D., and Burny, A. (1979). Humoral antibody response to bovine leukemia virus infection in cattle and sheep. *Cancer Res.* **39**, 1118-1123.
3. Chow, D. A., Greene, N. T., and Greenberg, A. H. (1979). Macrophage-dependent, NK-cell-independent "natural" surveillance of tumors in syngeneic mice. *Int. J. Cancer* **23**, 788-797.
4. Essex, M., Sliski, A., Cotter, S. M., Jakowski, R. M., and Hardy, W. D. Jr. (1975). Immunosurveillance of naturally occurring feline leukemia. *Science* **190**, 790-792.
5. Habu, S., Fukui, H., Shimamura, K., Kasai, Y., Okumura, K., and Tamaoki, N. (1980). *In vivo* effects of anti-asialo GM₁. I. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. *J. Immunol.* **127**, 34-38.
6. Honma, T., Onuma, M., Mikami, T., and Izawa, H. (1980). Bovine leukemia virus infection in Japan; Antibody and virus detection in cattle. *Jpn. J. Vet. Sci.* **42**, 5-8.
7. Honma, T., Onuma, M., Suneya, M., Mikami, T., and Izawa, H. (1980). Cytotoxic antibody in cattle and sheep exposed to bovine leukemia virus. *Arch. Virol.* **66**, 293-299.
8. Kodama, H., Onuma, M., Matsumoto, K., Yamamoto, S., Naiki, M., Mikami, T., and Izawa, H. Host immune responses in nude mice transplanted with bovine leukemia cell line (FLK). In: *Fifth International Symposium on Bovine Leukosis*. In press.
9. Olson, C. (1974). Bovine lymphosarcoma (leukemia)—a synopsis. *J. Am. Vet. Med. Assoc.* **165**, 630-632.
10. Onuma, M., Ishihara, K., Ohtani, T., Honma, T., Mikami, T., and Izawa, H. (1979). Seroepidemiological survey on antibodies against bovine leukemia virus in Japanese black cattle. *Jpn. J. Vet. Sci.* **41**, 601-605.
11. Onuma, M., Matsumoto, K., Mikami, T., and Izawa, H. (1979). Heterotransplantation of natural bovine lymphosarcoma and established cell lines to nude mice. In: *Advances in Comparative Leukemia Research*. (Yohn, D. S., Lapin, B. A., and Blackeslee, J. R. eds.), 43-44. Elsevier North Holland, New York.
12. Onuma, M., Matsumoto, K., Moriguchi, R., Fujimoto, Y., Miyake, Y., Mikami, T., and Izawa, H. (1981). Transformed phenotypes in long-term cultures persistently infected with bovine leukemia virus. *Can. J. Comp. Med.* **45**, 154-158.
13. Onuma, M., Watarai, S., Mikami, T., and Izawa, H. (1982). Cell surface antigens on sheep fibroblasts transformed by bovine leukemia virus. In: *Fourth International Symposium on Bovine Leukosis*. (Straub, O. C. ed.), 34-35. Martinus Nijhoff, Hague.
14. Onuma, M., Yamamoto, S., Hamada, K., Ogawa, Y., Kodama, H., Mikami, T., and Izawa, H. Mechanisms of prevention of bovine leukosis by immune responses. In: *Fifth International Symposium on Bovine Leukosis*. In press.
15. Santoni, A., Herberman, R. B., and Holden, H. T. (1979). Correlation between natural and antibody-dependent cell-mediated cytotoxicity against tumor targets in the mouse. I. Distribution of the reactivity. *J. Natl. Cancer Inst.* **62**, 109-116.
16. Suneya, M., Onuma, M., Mikami, T., and

- Izawa, H. (1982). Humoral and cellular immune responses to bovine leukemia virus infection in sheep. In: Fourth International Symposium on Bovine Leukosis. (Straub, O. C. ed.), 77–89. Martinus Nijhoff, Hague.
17. Takashima, I., and Olson, C. (1980). Bovine leukosis virus in sheep. Lymphocyte modification and surface-immunoglobulin-bearing cell numbers. *Vet. Microbiol.* **5**, 1–12.
18. Yamamoto, S., Onuma, M., Kodama, H., Mikami, T., and Izawa, H. Suppression of natural cytotoxic activity of lymphocytes from cattle and sheep during the progress of bovine leukosis. *Vet. Microbiol.* in press.

要 約

牛白血病由来株化細胞 (FLK) 接種によるヌードマウスの K 細胞活性の抑制: 山本慎一・小沼 操・児玉 洋・見上 彪・伊沢久夫・松本耕三¹⁾ (北海道大学獣医学部家畜伝染病学講座, ¹⁾医学部附属動物実験施設)——ヌードマウスに牛白血病由来株化細胞 (FLK) 生細胞をあらかじめ接種すると, 2 回目に接種した FLK 細胞の排除に遅延がみられた. FLK 細胞に対する, ヌードマウス脾の NK 細胞および K 細胞活性を 8 時間の ⁵¹Cr 遊離試験で調べると, FLK 生細胞接種ヌードマウスでは FLK 細胞に対する K 細胞活性が顕著に低下していた. しかし FLK および YAC-1 細胞に対する NK 細胞活性には差が見られなかった. 一方, グルタル・アルデヒド不活化 FLK 細胞あるいは, 乳剤にした FLK 死細胞を接種したヌードマウスでは FLK 細胞に対する NK および K 細胞活性に変化を生じなかった. これらの結果は, FLK 生細胞接種によりヌードマウスの K 細胞活性が抑制されて FLK 細胞排除が遅延することを示唆しており, 腫瘍増殖を促進するものと考えられた.