Properties of Tumor Infiltrated Cells Induced by N-CWS

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ABSTRACT. Analysis of surface marker of cells after intratumor injection with *Nocardia rubra* cell wall skeleton (N-CWS) resulted in gradually increasing percentage of macrophage, Pan T and BoCD4⁺ cells. Proportion of BoCD8⁺ cells gradually increased from 4th day and then decreased from 8th day after the injection. Fresh tumor infiltrated cells obtained from lymphatic nodule at 8 days after injection of N-CWS showed cytotoxic activity against bovine leukemia cell line, but this activity decreased with the time of cultivation and no activity could be detected after 14 days cultivation. These cultured cells were injected twice to lymphatic nodule at one week interval for adoptive immunotherapy and found to induce complete regression of nodule after 5 weeks from first injection.—KEY WORDS: bovine leukemia, immunotherapy, N-CWS, tumor infiltrated lymphocyte.

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Recent advances in adoptive immunotherapy (AIT) have used cytotoxic lymphocyte with broad antitumor reactivity [20]. Cultured tumor infiltrated lymphocytes (TIL) with interleukin 2 which are non-major histocompatibility complex (MHC) restricted killer cells have recently gained much attention because of their potential clinical application in cancer therapy.

Previously, we found a good therapeutic effect in intratumor injection of *Nocardia rubra* cell wall skeleton (N-CWS) in pre-leukemic cattle of enzootic bovine leukemia (EBL) [17]. The major population of the infiltrated cells were macrophage and T cells [25]. In present experiment, we analyzed lymphocyte phenotype of TIL induced by N-CWS in more detail. Moreover, by using cultured TIL obtained from tumor nodule treated with N-CWS, we applied AIT for a lymphatic nodule of preleukemic cattle in EBL to know the antitumor effect of TIL.

MATERIALS AND METHODS

Animals: A BLV-infected Holstein Friesian cow was used. The cow had some enlarged subcutaneous lymphatic nodules. The cow showed no clinical signs of enzootic bovine leukemia (EBL), however, it had tumor associated antigen (TAA) on peripheral blood lymphocyte and enlarged subcutaneous lymphatic nodules detected by complement dependent antibody cytotoxicity (CDAC) test using c143 monoclonal antibody against TAA of EBL [1]. The nodules showed follicular hyperplasia which had been considered to be initial lesion of EBL [18].

Histopathological observation: Lymphatic nodules were fixed in 10% buffered formalin, embedded in paraffin and stained hematoxylin and eosin.

Isolation and cultivation of TIL: Lyophilized N-CWS which was kindly provided by Dr. I. Azuma, Hokkaido University (Sapporo), was dispersed in phosphate buffered saline (pH 7.2) at a concentration of 1

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mg/ml just before use. Three lymphatic nodules were injected with 1 mg of N-CWS intralesionally, and the nodules were surgically removed at 1, 4 and 8 days after the injection. Non-injected nodules as control were also removed. Single cell suspensions from test nodule which was removed at 8 days after injection of N-CWS, were mixed with c143 monoclonal antibody plus rabbit complement to kill the TAA-positive cells. Viable TIL were separated by Ficoll-Conray method [5]. Mouse cell line L23 produces IL-2 that can maintain bovine IL-2 dependent cell line in our laboratory. The TIL (1×10^6 /ml) were cultured for 14 days in RPMI 1640 medium with 10% fetal calf serum, antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin and kanamycin) and 10% L23 supernatant and used for AIT.

Adoptive immunotherapy: An enlarged subcutaneous nodule with a mean diameter of 18.0 mm on the reverse cervical region of N-CWS injected site was used for AIT. The nodule was injected with 1×10^6 cultured TIL induced by N-CWS in 1 ml medium without IL-2, and one week later, 5×10^6 of TIL were also injected. Cultured TIL (1×10^7) obtained from nodule without injection of N-CWS were inoculated to other nodules 3 times at one week interval as control. After injection, tumor sizes were regularly measured with calipers.

Determination of lymphocyte phenotype: The composition of lymphocyte populations was monitored by fluorescence antibody assay using the monoclonal antibodies BAQ82A (pan T), CACT83A (BoCD4),

CACT80C(BoCD8), CH137A (macrophage), BAS9A (B cell) [6] and c143 (TAA positive cell in EBL) [1].

Cell mediated cytotoxicity: A 4 hr ^{51}Cr release assay was performed using the Ku-8 (EBL B cell line) as target cells [25]. Ku-8 cells were kindly supplied by Dr. H. Koyama (Kitasato Univ.). At 100:1 effector: target ratio, the cells were incubated for 4 hr at 37°C in a humidified 5% CO_2 atmosphere. Spontaneous ^{51}Cr release was determined by incubation of target cells without effector cells, and maximum release was determined by incubation of target cells with Triton X. The percentage of cytotoxicity was determined using the following formula;

$$\% \text{ of cytotoxicity} = \frac{(\text{experimental cpm} - \text{spontaneous cpm})}{(\text{maximum cpm} - \text{spontaneous cpm})} \times 100$$

RESULTS

TIL obtained from nodule without injection of N-CWS do not express any cytotoxic activity *in vivo*. For this reason, we applied AIT using TIL induced by N-CWS.

Before the application of AIT, we analyzed cell surface markers of the isolated TIL populations using monoclonal antibodies against T cell subsets, B cell, and c143 (Table 1). Most of the cells obtained from pretreated lymphatic nodules were B cell and TAA positive cell (>99.0%). One day

Table 1. Changes of cells in tumors with days after treatment

Days after injection of N-CWS	Percentage of cells in tumor					
	Pan T	BoCD4 ⁺	BoCD8 ⁺	Macrophage	B cell	TAA ⁺
Pretreatment	2.1	0.1>	0.1>	1.0>	99.0<	99.0<
1	5.3	1.6	2.1	0.5	95.9	60.0
4	20.4	5.8	14.1	3.5	63.0	38.0
8	37.2	27.4	2.3	13.2	64.0	38.1

Table 2. Changes of cytotoxic activity and surface marker with days of cultivation

Days of culture	% lysis ^{a)}	Percentage of surface marker of cultured cell			
		Pan T	BoCD4 ⁺	BoCD8 ⁺	Macrophage
0	19.8	37.2	27.4	2.3	13.2
7	1.2	82.1	79.3	40.2	1.0
14	0	99.0	99.0	95.0	0

a) effector: target=100 : 1.

after treatment, TAA positive cells decreased to 60.0% but the percentage of B cell remained constant (95.9%), while that of T cell slightly increased when compared to that of pretreated cells. Four days later, the percentages of B cell and TAA-positive cells decreased, while those of T cell and macrophage increased. A high proportion of T cell showed mainly BoCD8⁺ phenotype (14.1%). Eight days after treatment, percentages of T cell and macrophage increased, and the major T cell population changed to BoCD4⁺ (27.4%).

TIL obtained from lymphatic nodule 8 days after N-CWS treatment were cultured *in vitro* with IL-2 and compared cell surface markers and cytotoxic activity before and after cultivation (Table 2). Cytotoxic activity could be detected in fresh TIL however no activity could be detected in culture cells after 14 days cultivation. Almost all cells showed the surface markers of pan T, BoCD4 and BoCD8 positive after 14 days cultivation.

An enlarged subcutaneous nodule with a mean diameter of 18.0 mm was injected with cultured TIL induced by N-CWS (Fig. 2a). As shown in Fig. 1, remarkable decrease of the nodule size was evident after the second injection, and the nodule disappeared by 35 days after 1st inoculation (Figs. 2b and 2c). On the other hand, a subcutaneous nodule, which was injected with cultured TIL obtained from lymphatic nodule without injection of N-CWS as control, was not changed for observation period (Fig. 2d).

Fig. 1. Changes of mean diameter of tumor injected by cultured TIL cells. The tumor received 1×10^6 cultured cells ($\blacktriangledown$) for the first injection and 1 week later 1×10^7 of the same cells ($\blacktriangledown$) for the second injection.

DISCUSSION

The TIL isolated from solid tumors and expanded by IL-2 were reported to be 50 to 100 times more effective in lysis of autologous tumor cells than IL-2 expanded spleen cells from the same tumor-bearing mice [20]. Thus we tested AIT by using TIL obtained from lymphatic nodules of pre-leukemic cattle in EBL. The TIL showed no cytotoxic activity and the TIL cultivated with IL-2 also had no cytotoxic activity against EBL-B cell line (data not shown). Moreover AIT using these TIL (1×10^7 /injection, 3 times) showed no therapeutic effect to bovine tumors indicating that TIL for AIT is not effective for bovine system.

Fig. 2. Observations of nodules injected with cultured TIL at pre-injection (a), 35 days (b and c) and 50 days (d) after first inoculation.

- a) Before injection of TIL induced by N-CWS.
- b) After injection of TIL induced by N-CWS at 35 days.
- c) Cultured TIL used were induced by N-CWS. ($\times 220$)
- d) Cultured TIL used were obtained from lymphatic nodule without injection of N-CWS. ($\times 90$)

TIL often show various immunosuppressions in assays of cytotoxicity, natural killing, and proliferation [2, 3, 10, 15, 16, 21, 23, 24]. From these reports, we speculate that TIL obtained lymphatic nodules might be inhibited by immunosuppressive factors in assays of killer activity in this bovine system. Hence, it is necessary to activate the subpopulation of TIL for use in AIT.

Previously, we found the antitumor effect of N-CWS in five BLV-positive cattle with enlarged subcutaneous lymphatic nodules by injection of N-CWS into tumors [17]. We found infiltration of T cells and macrophages in the injected lesions [25]. Thus

we tried to induce TIL by N-CWS. After intratumor N-CWS injection, changes of population in TIL were rapid. Macrophages and T cells increased and tumor cells decreased after the injection. TIL obtained from tumors 8 days after N-CWS injection showed cytotoxic activity against EBL B cell line. Cultured TIL induced by N-CWS showed efficient therapeutic effect when the cells were used as AIT.

Injection of N-CWS into tumor nodules in which most of cells were TAA⁺ cells and B cells before injection, resulted in a decrease of percentage of TAA⁺ cells and B cells and the percentage of TAA⁺ cells was less than

that of B cells, indicating an increase of TAA-negative B cells after the treatment. Antigenic modulation of tumor antigens is known in mouse B cell lymphoma under the natural conditions [12]. In human B lymphoma system, antigenic modulation has also been reported during immunotherapy [13]. Thus, the increase of TAA-negative B cells by N-CWS treatment might be caused by antigenic modulation and the modulated cells resulted in non-reactivity to c143 similar to that of rodent and human systems.

Infiltration of mononuclear cells into tumors is commonly observed in human and rodent systems, and most of TIL were found to have killer/suppressor phenotype [7, 9, 19]. A significant association has been reported between the prognosis of cancer patients and the degree of mononuclear cell infiltration in tumor [4, 11, 15, 22]. In the present study, tumor infiltrated mononuclear cells induced by N-CWS were macrophage, helper T cell (BoCD4⁺) and cytotoxic T cell (BoCD8⁺). When cultured TIL induced by N-CWS was injected into tumors for AIT, the regression of bovine tumor was observed (Fig.1). The population of the cultured TIL used for AIT was T cell with both BoCD4 and BoCD8 markers. Since these cultured TIL had no cytotoxic activity against bovine tumor cells *in vitro*, the cells may not be effector cells *in vivo* and the effector cells might be induced by injected TIL [8]. We could examine AIT only in one case in the present experiment, thus it is necessary to apply more cases to fully understand the antitumor mechanisms in bovine system.

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REFERENCES

1. Aida, Y., Onuma, M., Mikami, T., and Izawa, H. 1985. Topographical analysis of tumor-associated antigens on bovine leukemia virus-induced bovine lymphosarcoma. *Cancer Res.* 45: 1181-1186.
2. Akiyama, M., Bean, A. M., Sadamoto, K., Takahashi, Y., and Brankovan, V. 1983. Suppression of the responsiveness of lymphocytes from cancer patients triggered by co-culture with autologous tumor-derived cells. *J. Immunol.* 131: 3085-3090.
3. Alice, Y., Watts, H., Jaffe, N., and Perkman, R. 1977. Concomitant presence of tumor-specific cytotoxic and inhibitor lymphocytes in patients with osteogenic sarcoma. *New Engl. J. Med.* 55: 124-131.
4. Bennet, S. S., Futrell, J. W., Roth, J. A., Hoye, R. C., and Kechem, A. S. 1971. Prognostic significance of histologic host response in cancer of the larynx or hypolarynx. *Cancer* 28: 1255-1261.
5. Boyum, A. 1968. Isolation of leukocytes from human blood, further observations. Methylcellulose, dextran and ficoll as erythrocyte aggregating agents. *Scand. J. Clin. Lab. Invest.* 21: 31-50.
6. Davis, W. C., Marusic, S., Lewin, A. H., Splitter, A. G., Lance, E. P., Travis, C. M., and Gorham, R. J. 1987. The development and analysis of species specific and cross reactive monoclonal antibodies to leukocyte differentiation antigens and antigens of the major histocompatibility complex for use in the study of the immune system in cattle and other species. pp. 337-376. Elsevier Science Publishers B. V.
7. Evans, R. and Duffy, T. M. 1985. Amplification of immune T lymphocyte function in situ: The identification of active components of the immunologic network during tumor rejection. *J. Immunol.* 135: 1498-1504.
8. Greenberg, D. P., Cheever, A. M., and Fefer, A. 1981. Eradication of disseminated murine leukemia by chemotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt1⁺2⁻ lymphocytes. *J. Exp. Med.* 154: 952-963.
9. Ibayashi, Y., Ueda, T., Ueda, T., and Kikuchi, K. 1985. Functional analysis of mononuclear cells infiltrating into tumors: Differential cytotoxicity of mononuclear cells from tumors of immune and nonimmune rats. *J. Immunol.* 134: 648-653.
10. Jerrells, R. T., Dean, H. J., Richardson, L. G., McCoy, L. J., and Herberman, B. R. 1978. Role of suppressor cells in depression of *in vitro* lymphoproliferative responses of lung cancer and breast cancer patients. *J. Natl. Cancer Inst.* 61: 1001-1009.
11. Kikuchi, K., Ishii, Y., Ueno, H., and Koshiba, H. 1976. Cell mediated immunity involved in autochthonous tumor rejection in rats. *Ann. New York Acad. Sci.* 276: 188-194.

12. Levy, R. 1985. Biologicals for cancer treatment: Monoclonal antibodies. *Hospital Practice* 15: 67-74.
13. Meeker, T., Lowder, J., Cleary, M., Stewart, S., Warnke, R., Sklar, J., and Levy, R. 1985. Emergence of idiotype variants during treatment of B-cell lymphoma with anti-idiotypic antibodies. *New Engl. J. Med.* 26: 1658-1665.
14. Moore, O. S. Jr. and Foote, F. W. Jr. 1949. The relatively favorable prognosis of medullary carcinoma of the breast cancer. *Cancer* 2: 635-641.
15. Moore, M. and Vose, M. B. 1981. Extravascular natural cytotoxicity in man: anti-k562 activity of lymph-node and tumor infiltrating lymphocytes. *Int. J. Cancer* 27: 265-272.
16. Moy, M. P., Holmes, C. E., and Golub, H. S. 1985. Depression of natural killer cytotoxic activity in lymphocytes infiltrating human pulmonary tumors. *Cancer Res.* 45: 57-60.
17. Onuma, M., Yamamoto, M., Yasutomi, Y., Kawakami, Y., and Azuma, I. 1989. Regression of bovine lymphosarcoma by treatment with cell-wall skeleton of *Nocardia rubra*. *Vaccine* 7: 121-124.
18. Onuma, M., Yasutomi, Y., Okada, M.H., Matsukawa, K., Yoshikawa, H., Yoshikawa, T., Okada, K., Takahashi, K., Kirisawa, R., and Kawakami, Y. 1988. The screening of cattle with potential for developing leukemia by using monoclonal antibody against bovine leukemia cells. *Jpn. J. Cancer Res. (Gann)* 79: 275-281.
19. Rabinowich, H., Cohen, R., Bruderman, I., Steiner, Z., and Klajman, A. 1978. Functional analysis of mononuclear cells infiltrating into tumors: lysis of autologous human tumor cells by cultured infiltrating lymphocytes. *Cancer Res.* 47: 173-177.
20. Rosenberg, S. A., Spiess, P., and Lafreniere, R. L. 1986. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* 233: 1318-1321.
21. Roth, A. J., Grimm, A. E., Gupta, K. R., and Ames, S. R. 1982. Immunoregulatory factors derived from human tumors. I. Immunologic and biochemical characterization of factors that suppress lymphocyte proliferative and cytotoxic response *in vitro*. *J. Immunol.* 128: 1955-1962.
22. Shimokawara, I., Imamura, M., Yamanaka, N., Ishii, Y., and Kikuchi, K. 1982. Identification of lymphocytes subpopulation in human breast cancer tissue and its significance; an immunoperoxidase study with anti-human T- and B-cell sera. *Cancer* 49: 1456-1462.
23. Vose, M. B. and Moore, M. 1979. Suppressor cell activity of lymphocytes infiltrating human lung and breast tumors. *Int. J. Cancer* 24: 579-585.
24. Whitehead, S. J. and Kim, S. Y. 1980. An inhibitor of lymphocyte proliferation produced by a human colonic adenocarcinoma cell line in culture. *Cancer Res.* 40:29-35.
25. Yasutomi, Y., Onuma, M., Yamamoto, Y., Kawakami, Y., and Azuma, I. 1989. *In vitro* activation of bovine macrophage and peripheral blood mononuclear cells by *Nocardia rubra* cell wall skeleton (N-CWS). *J. Vet. Med. B.* (in press)

要 約

N-CWS 接種により誘導される腫瘍内浸潤細胞の分析：保富康宏・小沼 操・William C. Davis¹⁾・川上善三 (酪農学園大学獣医微生物学教室, ¹⁾Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University)——牛白血病腫瘍内に *Nocardia rubra* cell wall skeleton を接種したところマクロファージ, PanT および BoCD 4⁺細胞の増加が認められた。BoCD 8⁺細胞は接種後4日目まで徐々に増加したが, 8日目では減少した。接種後8日目の浸潤細胞は牛白血病細胞株に対し細胞障害活性を持っていたが, 培養に伴いその活性は減少し培養14日目で消失した。この細胞障害活性を持たない細胞を他の腫瘍内に1週間隔で2回接種(養子免疫)したところ初回接種後5週でその腫瘍は消失した。