

Phenotypic Analysis and Effects of Sequential Administration of Activated Canine Lymphocytes on Healthy Beagles

Yuki HOSHINO^{1)*}, Satoshi TAKAGI¹⁾, Tomohiro OSAKI²⁾, Masahiro OKUMURA¹⁾ and Toru FUJINAGA¹⁾

¹⁾Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences and ²⁾Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido 060-0818, Japan

(Received 21 December 2007/Accepted 7 February 2008)

ABSTRACT. We attempted to accumulate the basic data for evaluation of activated lymphocyte therapy for small animal medicine. The peripheral blood mononuclear cells (PBMCs) collected from healthy dogs were activated using anti-CD3 antibody and human recombinant (hr) interleukin (IL)-2 and reactivated using hr interferon (IFN)- α and hr IL-2. The property of obtained cells was compared with PBMCs. The number of cells was shown to have increased approximately > 50 -fold by cultivation. The proportion of CD8⁺ cells was significantly increased, the cytotoxicity of the cultured cells was revealed to have been reinforced. Additionally, CD56 mRNA levels tended to have increased. The cells obtained by this method were confirmed to be activated lymphocytes. Furthermore, we investigated the effects of sequential administration of the obtained cells to healthy dogs. By sequential administration of the activated lymphocytes, the cell proliferative activity, proportion of CD4⁺ cells and CD8⁺ cells, and serum IFN- γ concentration were shown to have increased, and no severe adverse effects were observed. Consequently, activated lymphocytes could be induced using anti-CD3 antibody and IL-2 in healthy dogs, and sequential administration of activated lymphocytes reinforced the recipient's immunity.

KEY WORDS: activated lymphocytes, canine, cytokine, tumor immunotherapy.

J. Vet. Med. Sci. 70(6): 581–588, 2008

The longevity of dogs has increased due to improvements in diets, breeding environments, and veterinary medical technology; however, this has resulted in an increase in the incidence of canine cancer [8]. Currently, cancer is treated by surgery, radiotherapy, chemotherapy, or a combination of these therapies [3]. However, these therapies often cause severe adverse effects [42]. Therefore, the demand for immunotherapy that has few adverse effects in veterinary [2] and human medicine has increased as an adjuvant therapy.

Antitumor therapy using activated lymphocytes is a form of passive immunotherapy that involves administration of autologous lymphocytes that have been proliferated by stimulation and culture with certain cytokines [18, 32]. This form of immunotherapy is expected to (1) trigger the cytotoxic activity of the administered lymphocytes against target tissues, and (2) indirectly induce cell-mediated immunity by activation of T lymphocytes and natural killer (NK) cells that are stimulated by various cytokines secreted from the administered cells [27].

Generally, most of animal tumors are diagnosed in the later stages, and some malignant tumors have a rapidly progressive behavior [41]. In tumor-bearing animals, antitumor immunity is expected to be suppressed by the growing tumor through various mechanisms [23, 29, 30, 36]. In such cases, spontaneous activation of cellular immunity against the tumor appears to be restricted. In such immunosuppressed animals, activated lymphocyte therapy by adminis-

tration of autogenous lymphocytes proliferated and activated *ex vivo* is supposed to be fairly effective. This therapy is, therefore, applicable to small animal cancers owing to its immunoenhancing properties.

Some factors, including tumor stage and general physical condition of the animal, affect lymphocyte activation. This may lead to confusion in understanding the precise mechanism of activated lymphocyte therapy. A suitable method for evaluation of the immunoenhancing effects of immunotherapy has not been established yet. In humans, most of the cells obtained by cultivation of activated lymphocytes are reported to be cluster of differentiation (CD)-3⁺ T or NK cells [17], and the cytotoxic activity of these cells has been proved to increase after cultivation [40]. When canine lymphocytes were cultured with anti-CD3 antibody and human recombinant (hr) interleukin (IL)-2, the number of lymphocytes was observed to have increased [24]. And in the previous report, hr interferon (IFN)- α and hr IL-2 were used for reactivation in order to activate NK cells [10]. Therefore, these two protocols were combined in this study. There was no report on the changes in the cytotoxic activity of these cells and the effect of sequential administration of these cells as activated lymphocyte therapy.

In previous reports, canine CD56—one of the markers of human NK cells [37]—mRNA increased in stimulated lymphocytes [4, 21]. The usage of relative quantification CD56 mRNA method was investigated during activated lymphocyte therapy in this study.

This study was designed to accumulate the basic data for evaluation of activated lymphocyte therapy including examination of a population of activated lymphocytes using anti-CD3 antibody, IL-2 and IFN- α and determination of the cytotoxic activity of these activated cells.

* CORRESPONDENCE TO: HOSHINO, Y., Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences Graduate School of Veterinary Medicine Hokkaido University, Kita-18 Nishi-9 Sapporo, Hokkaido 060-0818, Japan.
e-mail: yukito@vetmed.hokudai.ac.jp

MATERIALS AND METHODS

Experimental animals

This study was performed on 6 clinically normal beagles (4 males and 2 females; age, 18–26 months; body weight, 10–14 kg). This research was conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Graduate School of Veterinary Medicine, Hokkaido University. Our experimental protocols were approved by the Institutional Animal Care and Use Committee of our institution.

Preparation of activated lymphocytes

Separation of peripheral blood mononuclear cells (PBMCs): Peripheral blood samples from the dogs were heparinized (10 U/ml) and were diluted to twice their original volume with physiological buffer saline (PBS). PBMCs were isolated by Ficoll-Paque density gradient centrifugation (specific gravity: 1.077) [5, 26]. For further use, PBMCs were adjusted to a final concentration of 1×10^6 cells/500 μ l of PBS and then separated into microtubes. One of the microtubes was centrifuged (at $5,800 \times g$ for 1 min at 22°C), and the supernatant was removed. The microtubes were tapped and stored at –20°C until RNA extraction. Three of the microtubes that had been prepared for phenotypic assay were stored at 4°C.

Culture of PBMCs: PBMCs were cultured according to previously reported methods [9, 24] with some modifications. The summarized procedure is as follows. A 75 cm² culture flask was incubated for 24 hr at room temperature with a mixture of 25 μ l anti-CD3 antibody (1 mg/ml mouse anti-canine CD3; Serotec, Oxford, U.K.) in 5 ml physiological saline (final concentration, 5 μ g/ml). PBMCs were cultured in a complete medium (CM; containing 700 RU/ml of IL-2) [comprising RPMI1640+9 (Nikken Biomedical Lab, Kyoto, Japan), RPMI1640 (GIBCO PBL, Gaithersburg, NY, U.S.A.), and fetal bovine serum (FBS; Moregate Biotech, Bulimba, Australia) in a ratio of 7:2:1]. On day 0, PBMCs were adjusted to a final concentration of 3×10^7 cells/10 ml of CM. The PBMC suspension was incubated in an anti-CD3 antibody-coated flask at 37°C under 5% CO₂. On days 4, 8, and 12 after incubation, CM was added and the number of live cells was counted by the trypan blue dye exclusion method. The additional medium used after day 8 was CM-2 (containing 175 RU/ml of IL-2 and 2% FBS). If cell growth was remarkable, a gas-permeable culture bag (Nipro, Osaka) was used. On day 14, the cells were harvested and centrifuged (at $850 \times g$ for 10 min at 22°C), and the supernatant was removed. Further, 15 ml of RPMI1640+9 and IFN- α (15 U of hr IFN alpha A; PBL Biomedical Laboratories, Piscataway, NJ, U.S.A.) were added to the cell pellets. These cells were incubated at 37°C under 5% CO₂ for 15 min, washed twice, and resuspended with PBS.

Comparison of PBMCs and activated lymphocytes

Phenotype assay: To both microtubes, 1×10^6 PBMCs

were added. The microtubes were centrifuged (at $400 \times g$ for 3 min at 4°C), and the supernatants were discarded. Ten microliters of fluorescent isothiocyanate (FITC)-labeled anti-IgG antibody (goat anti-mouse IgG, rat absorbed; Serotec Ltd.) was added to one of the tubes. Ten microliters each of FITC-labeled anti-CD4 antibody (rat anti-canine CD4; Serotec Ltd.) and R-phycoerthrin (R-PE)-labeled anti-CD8 antibody (rat anti-canine CD8; Serotec Ltd.) were added to the sediment in the other tube, and the tube was tapped. The samples were placed on ice, reacted for 30 min, and washed 3 times with ice-cold PBS. The samples were resuspended in 500 μ l of PBS. Phenotyping of the lymphocytes was performed by flow cytometric analysis (Beckman Coulter Inc., Fullerton, CA, U.S.A.).

Cytotoxic assay: For determination of natural cytotoxicity, non-adherent PBMCs and activated lymphocytes were used. All cells were incubated at 37°C under 5% CO₂.

(1) Culture of tumor cells

Four malignant melanoma cell lines (CMec, CMM-1, CMM-2, and LMec) were used [22]. Each line was cultured with RPMI1640 culture medium containing 10% FBS, 10 U/ml penicillin G potassium, 0.1 mg/ml streptomycin, and 10 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid.

(2) Rose Bengal assay

For determining natural cytotoxicity, a colorimetric Rose Bengal assay (RBA) was used [16]. Target (T) cells (melanoma cell lines) were aliquoted in 96-well flat-bottomed plates at a final concentration of 1×10^5 viable cells in 100 μ l of RPMI1640 medium (10% FBS) per well and incubated for 8 hr. Thereafter, 100 μ l of effector (E) cells (PBMCs and activated lymphocytes) were added to each well, resulting in E/T ratios of 100:1, 50:1, 25:1, and 12.5:1. For negative control, 100 μ l RPMI1640 medium was added instead of the effector cells. All tests were run in triplicate. After incubation for 14 hr, the suspended cells were removed. The remaining adherent target cells were exposed to Rose Bengal (Wako, Osaka) for 3 min. After 3 washes, Rose Bengal was dissolved with 50% ethanol/PBS, and the optical density (OD) was measured using a microplate reader (MTP-120, Corona Electric, Ibaraki) at a wavelength of 562 nm. Cytotoxicity was calculated according to the formula used by Funk *et al.* [14]:

$$\% \text{ cytotoxicity} = 100 - (\text{OD}_{\text{test}} / \text{OD}_{\text{control}} \times 100)$$

(3) Morphological examination

Target cells (CMec) were adjusted to a final concentration of 1×10^5 cells/100 μ l of the RPMI1640 medium. Further, 150 μ l of each target cell was plated onto 16-well chamber slides (IWAKI, Tokyo). After incubation for 8 hr, the effector cells (PBMCs and activated lymphocytes) were added resulting in E/T ratios of 100:1 and 50:1. After incubation for 14 hr, each medium was removed, washed once with PBS, and fixed with methanol. Each slide was stained with Giemsa solution.

Relative quantification of CD56 mRNA expression in PBMCs and activated lymphocytes: Real-time polymerase chain reaction (PCR) was performed for relative quantifica-

tion of CD56 mRNA expression in PBMCs and activated lymphocytes. RNA was extracted by using RNeasy Mini Kits (Qiagen, Les Ulis, France) according to the manufacturer's instructions. cDNA was synthesized according to a previously reported method [21]. Relative quantification was performed by a previously reported method in order to determine the level of CD56 mRNA expression [21].

Sequential administration of activated lymphocytes to normal dogs

This procedure was performed on 3 clinically healthy male beagles (body weight, 13.3–13.7 kg; all having the same blood group; age, 18 months). The day of the first administration of activated lymphocytes was considered as day 0, and on days -14, 0, 14, and 28, we collected 20 ml of blood for culture of PBMCs. The 14th day cultured activated lymphocytes were administered intravenously to the appropriate dogs with autologous serum on days 0, 14, 28, and 42. The physical condition of these dogs was examined for 3 day to observe for adverse effects. Furthermore, 1×10^6 activated lymphocytes were separated and evaluated for relative quantification of CD56 mRNA expression and phenotype assay performed on days -14, 7, 14, 21, 28, 35, 42, 49, 56, and 70. For quantitative determination of canine IFN- γ concentration, we collected the culture supernatant on every 14th day and the serum sample on days 0, 7, 14, 21, 28, 35, 42, 49, 56, and 70. For detecting of IFN- γ concentration, a Canine IFN- γ immunoassay kit (R&D systems Inc., Minneapolis, MN, U.S.A.) was used.

Additionally, as a test for sterility of the culture solution, the culture supernatant on the 14th day was examined for endotoxin concentration by the synthetic chromogenic substrate method and for mRNA expression by using the TaKaRa PCR Mycoplasma Detection Set (TAKARA BIO INC., Shiga).

Statistical analysis

All results are represented as mean \pm standard error. For statistical analysis, the Wilcoxon signed-ranks test was used, and values of $p < 0.05$ were considered statistically significant.

RESULTS

Number of activated lymphocytes

The number of PBMCs obtained from the 6 normal dogs was $1.10\text{--}3.00 \times 10^7$ cells (average, 2.18×10^7 cells) on isolation; however, after culture, it increased to $0.51\text{--}2.20 \times 10^9$ cells (average, 1.16×10^9), *i.e.*, an 18.3- to 112.0-fold (average, 57.4-fold) increase (data not shown).

Comparison of PBMCs and activated lymphocytes

Phenotype assay: To clarify the cellular phenotype of PBMCs and activated lymphocytes, flow cytometric analysis was performed. In PBMCs, the gate of lymphocytes was set up by control. The proportion of lymphocytes was approximately 30%. In activated lymphocytes, all cells

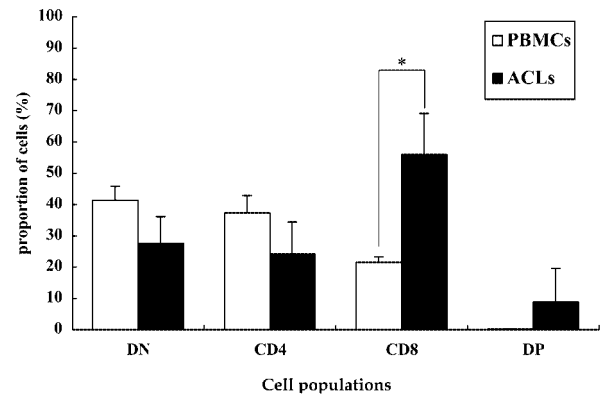


Fig. 1. The time-course changes in the proportions of PBMCs and activated lymphocytes ($n=5$). The proportions of CD4 $^-$ /CD8 $^-$ double negative (DN) cells and CD4 $^+$ cells decreased and the proportions of CD8 $^+$ cells and CD4 $^+$ /CD8 $^+$ double positive (DP) cells increased among the activated lymphocytes (ACLs) as compared to the peripheral blood mononuclear cells (PBMCs). The increase in the proportion of CD8 $^+$ cells was significant (*: $p < 0.05$). CD8 $^+$ cells are roughly regarded as cytotoxic T cells. All data are represented as mean \pm standard error.

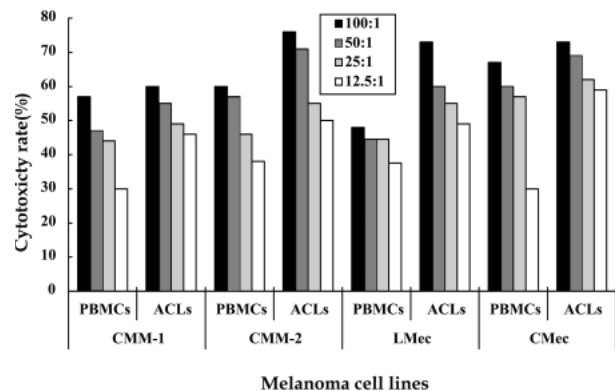


Fig. 2. The changes in the cytotoxicity of PBMCs and ACLs against melanoma cells. The cytotoxicity was calculated from the numbers of the effector cells (PBMCs and ACLs) and the target cells (4 different melanoma cell lines). ACLs show higher cytotoxicity than PBMCs in each cell line.

were regarded as lymphocytes, because the other cells in PBMCs had a short life compared to lymphocytes. A series of changes was observed in the proportion of PBMCs and activated lymphocytes obtained from the 6 normal dogs, as shown in Fig. 1. Among PBMCs, the proportion of CD4 $^+$ cells was predominance. In contrast, among the activated lymphocytes, the proportion of CD8 $^+$ cells was predominance. The increase in CD8 $^+$ cells among the activated lymphocytes was significant ($p < 0.05$). Additionally, among the activated lymphocytes, the proportion of CD4 $^+$ /CD8 $^+$ double positive (DP) cells increased, while that of CD4 $^-$ /CD8 $^-$ double negative (DN) cells decreased.

Analysis of the cytotoxic activity of the PBMCs and activated lymphocytes: Cytotoxicity assay was performed using

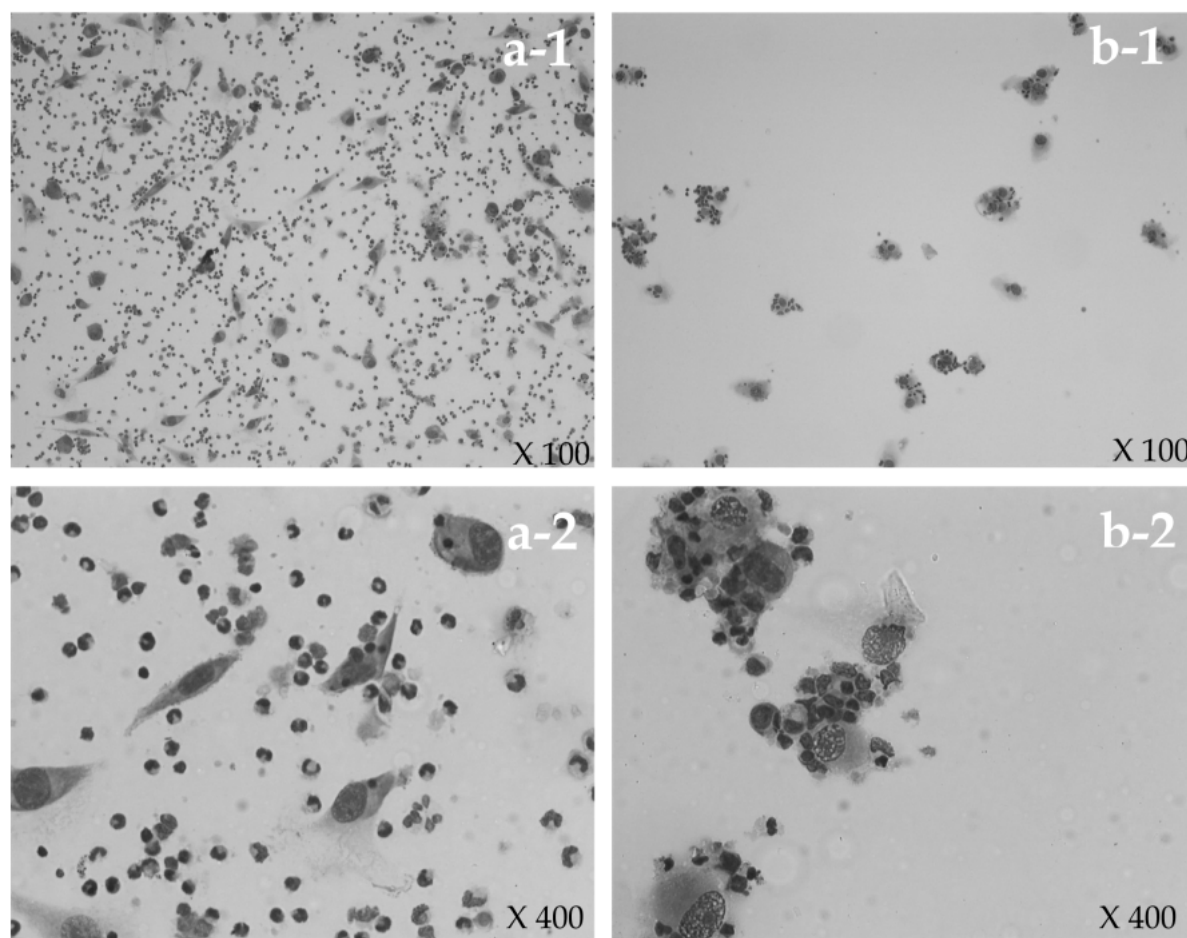


Fig. 3. Giemsa stained specimen of the cytotoxicity of PBMCs and ACLs against melanoma cells (E/T=50:1). Target cells are melanoma cell line (CMec). a. Effector cells: PBMCs. 1. $\times 100$: Many melanoma cells are alive. 2. $\times 400$: No PBMCs surrounding the melanoma cells. b. Effector cells: ACLs. 1. $\times 100$: Few melanoma cells are alive. 2. $\times 400$: ACLs surrounding the melanoma cells.

canine melanoma cells as the target cells. After coculture for 14 hr, activated lymphocytes showed higher cytotoxicity than PBMCs in each cell line (Fig. 2).

Morphological examination: Among PBMCs, most of the melanoma cells were alive (Fig. 3-a), and the PBMCs did not aggregate around the melanoma cells at E/T ratios of 100:1 and 50:1 (Fig. 3-a-2). In contrast, there were only a few live melanoma cells among the activated lymphocytes (Fig. 3-b), and the activated lymphocytes aggregated around the surviving melanoma cells (Fig. 3-b-2).

CD56 mRNA expression: To investigate for CD56-positive cells, relative quantification of CD56 mRNA expression was determined by real-time PCR. Although CD56 mRNA expression level of activated lymphocytes tended to increase, the difference was not significant (data not shown).

Influence of sequential administration of activated lymphocytes to normal dogs

The number of activated lymphocytes obtained was

observed to increase gradually: 1.92-, 2.18-, 5.69-, 9.38-fold after the first, second, third and fourth culture compared to the number of PBMCs, respectively. The phenotypic proportions of PBMCs and activated lymphocytes were investigated from the second to the fourth culture. The proportion of cells after the first culture was not determined because the number of cells obtained was very small. Figure 4 shows the average of phenotypic proportions after each culture cycle. Throughout the period of administration, the proportions of CD4⁺ cells and DN cells tended to have decreased, and the proportions of CD8⁺ cells and DP cells tended to have increased.

Relative quantification was performed to determine CD56 mRNA expression in PBMCs and activated lymphocytes. Figure 5 shows the average relative CD56 mRNA levels among the 3 dogs at each culture cycle. The increase in CD56 mRNA expression in activated lymphocytes was higher than that in PBMCs during the study period. CD56 mRNA levels at the first, second and fourth culture cycles were significantly higher than that in PBMCs ($p < 0.01$).

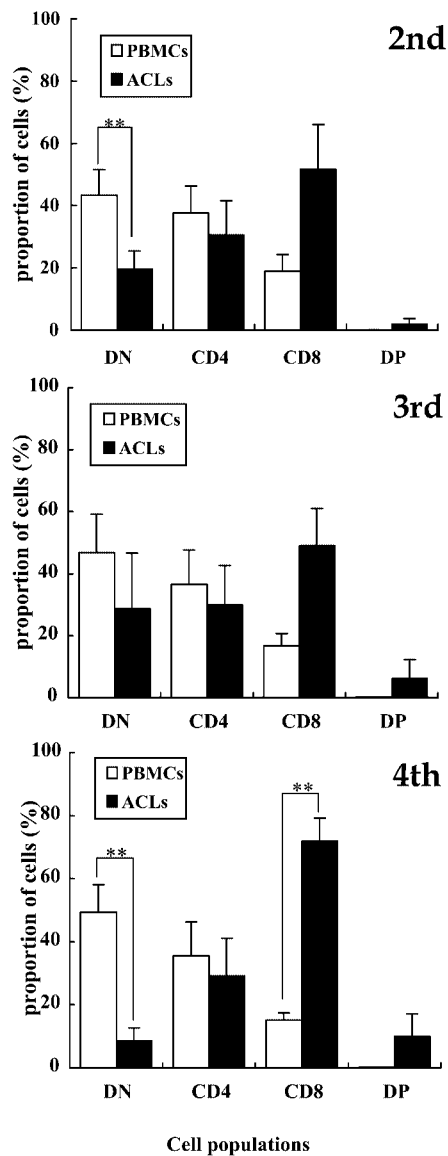


Fig. 4. The changes in the cell proportions among the PBMCs and ACLs during sequential administration of the ACLs ($n=3$). The figure shows the average cell proportions after every culture cycle: the second culture cycle (upper panel), third culture cycle (middle panel), and fourth culture cycle (lower panel). All of culture cycles show a decrease in the proportions of DN and CD4⁺ cells and an increase in the proportions of CD8⁺ cells and DP cells. In particular, the decrease in the proportion of DN cells at the second and fourth culture cycle and the increase in the proportion of CD8⁺ cells at the fourth culture cycle was significant (**; $p<0.01$). All data are represented as mean \pm standard error.

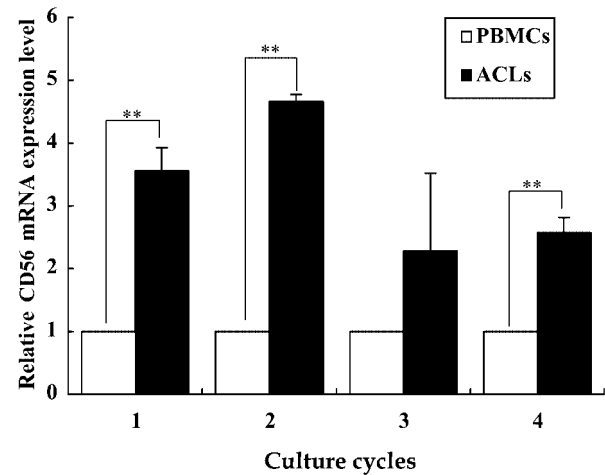


Fig. 5. CD56 mRNA expression levels in PBMCs and ACLs during sequential administration ($n=3$). The figure shows the relative CD56 mRNA expression levels, and the CD56 mRNA level in PBMCs was defined as 1.00. ACLs showed higher CD56 mRNA expression than PBMCs after every culture cycle. Particularly, CD56 mRNA levels at the first, second, and fourth cultures were significantly higher than that in PBMCs (**; $p<0.01$). All data are represented as mean \pm standard error.

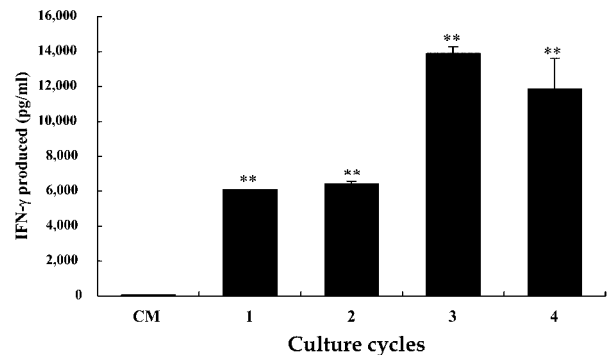


Fig. 6. IFN- γ production by ACLs ($n=3$). Interferon (IFN)- γ concentration in the culture supernatant was determined and was shown to be significantly high 4 times that of the control (**; $p<0.01$). All data are represented as mean \pm standard error.

The IFN- γ concentration in the activated lymphocyte culture supernatant on the 14th day was determined by enzyme-linked immunosorbent assay. Figure 6 shows the average of the IFN- γ concentration among the 3 dogs in terms of the number of times of cultivation. A significantly higher amount of IFN- γ was produced in the CM medium (control) at all times ($p<0.01$).

The biological response of the dogs after sequential administration of activated lymphocytes was examined. The time-course changes in the proportion of the PBMCs after administration was examined by flow cytometric analysis. The outcome of dog A, which showed the typical time-course attitude was exhibited in Fig. 7. On comparing the proportions of PBMCs on day 28 with that on day 35, a

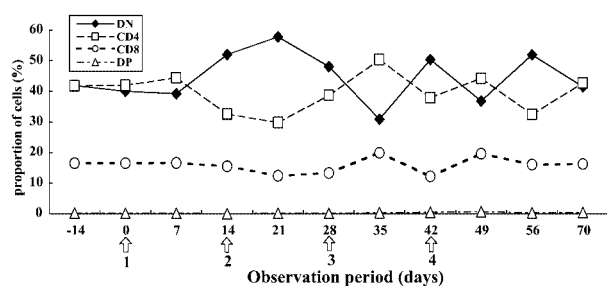


Fig. 7. The time-course changes in the cells proportions among the PBMCs during the observation period (dog A). White arrow showed a day of ACLs administration. From day 35 to day 49, a decrease in the proportion of DN cells and an increase in the proportions of CD4⁺ cells and CD8⁺ cells were noted. All of them returned to the respective baseline value after 1 week.

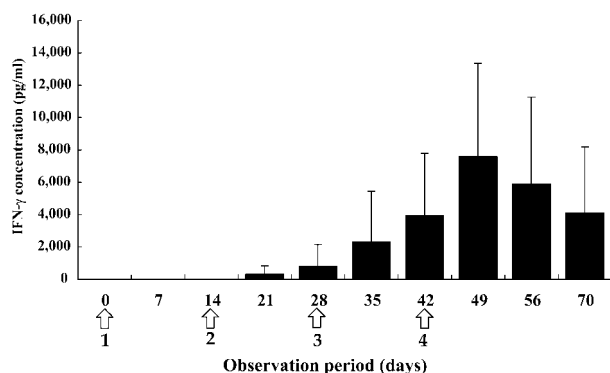


Fig. 8. Serum IFN- γ concentration in normal dogs ($n=3$). White arrow showed a day of ACLs administration. Serum IFN- γ concentration was hardly detectable until day 14; however, it increased gradually during sequential administration of activated lymphocytes from day 21 to day 49. Subsequently, it decreased gradually from day 56 to day 70, and reduced to approximately 50% of the peak value at 4 weeks after the last administration. All data are represented as mean \pm standard error.

decrease in the proportion of DN cells and an increase in the proportions of CD4⁺ cells and CD8⁺ cells were observed. A similar change was observed on days 42 and 49. In each instance, the altered values returned to the baseline ratio after 2 weeks. CD56 mRNA expression in the PBMCs was determined; however, no obvious change was found. Figure 8 shows the average serum IFN- γ concentration among the 3 dogs. Serum IFN- γ concentration was hardly detectable until day 14; however, it increased gradually during sequential administration of activated lymphocytes from day 21 to day 49. It then decreased gradually from day 56 to day 70 and reduced to approximately 50% of the peak values at 4 weeks after the last administration.

There were no adverse effects in clinical findings following sequential administration of activated lymphocytes in any of the experimental dogs during the study period. The endotoxin concentrations in the culture medium were within the normal limit (<5 pg/ml), and mycoplasma were not

detected in any of the samples.

DISCUSSION

As a trial of antitumor immunotherapy in veterinary patients, activated lymphocyte therapy was applied by administration of autogenous lymphocytes that had been proliferated and activated *ex vivo*. In human medicine, this type of immunotherapy is referred to as lymphokine-activated killer (LAK) therapy [34], CD3-LAK therapy, or BAK therapy [10]. Each clinical study has employed its own methods for application of growth factors to activated lymphocytes. Regardless of the growth factors used, the clinical results of activated lymphocyte therapy have been acceptable and favorable for controlling malignant tumors [12]. Activated lymphocyte therapy reduced the recurrence rate and lengthened the disease-free interval after surgical resection of hepatocellular carcinoma [38] and prolonged the life span without any adverse effects in patients with progressive solid tumors [11].

In veterinary medicine, activated lymphocyte therapy and dendritic cell-based vaccines are currently being paid much attention for clinical usage in tumor-bearing animals. Even after clinical trials, no investigation has revealed the clinical efficacy of activated lymphocyte therapy. Although many methods of lymphocyte activation have been reported, for example, concanavalin A [25]—a kind of mitogen, and low-dose IL-2 [19], the most frequently used growth factors are anti-CD3 antibody and IL-2 [24].

In present study, PBMCs isolated from canine blood were cultured with mouse anti-canine CD3 antibody and hr IL-2 for 14 day and were reactivated with hr IFN- α and hr IL-2 in order to activate NK cells [10].

The number of PBMCs increased approximately 57-fold after culture for 14 day. The proliferative ability was different in individual dogs as previously reported [24, 25]. Among the activated lymphocytes obtained at 14 day after culture, the proportions of CD8⁺ cells and DP cells were observed to have increased, as compared to the cell proportions among the PBMCs. The cytotoxicity of activated lymphocytes against melanoma cells was revealed to have been reinforced by cultivation as mentioned above. CD8⁺ cells are generally assumed to comprise a majority of cytotoxic T cells, and the cytotoxicity of these cells is restricted to the major histocompatibility complex (MHC) class I, which should be target specific and powerful [33]. DP cells contain immature lymphocytes that differentiate into CD4⁺ or CD8⁺ lymphocytes [39].

Signals mediated by some adhesion molecule may induce apoptosis of the melanoma cells surrounded by the activated lymphocytes [20]. The melanoma cells used here were established as a cell line, and their MHC specificity was completely different from that of the cultured lymphocytes. The cytotoxicity might have been mediated by MHC-unrestricted cells. Therefore, the candidate cells regulating the cells showing MHC-unrestricted cytotoxicity would be NK or NKT cells [1, 13, 28].

There were no severe adverse effects of sequential administration of activated lymphocytes in any of the experimental dogs during the study period. The number of activated lymphocytes obtained from the same amount of blood was likely to have increased with an increase in the administration of activated lymphocytes. Serum IFN- γ concentration and the proportion of CD8⁺ cells among the PBMCs were shown to have increased significantly with the administration of activated lymphocytes. This might indicate that administration of cultured CD4⁺ T cells promoted IL-2 secretion and induced T cell proliferation [31].

An increase in the proportions of CD4⁺ cells and CD8⁺ cells was evident within 7 days after the third and fourth administrations of activated lymphocytes. Serum IFN- γ concentration was also observed to have increased simultaneously. Administration of activated CD4⁺ cells and CD8⁺ cells would promote the secretion of IL-2 and IFN- γ , thus inducing proliferation and activation of T and NK cells, and further accelerating IFN- γ production [6, 7]. Serum IFN- γ concentration was increased first, and then, the cytotoxic ability of the PBMCs might have been enhanced, since IFN- γ promoted the activation of NK cells [35]. Serum IFN- γ concentration increased evidently during sequential administration of the activated lymphocytes [15], and it reduced to approximately 50% of the peak values at 4 weeks after the last administration. This fluctuation in serum IFN- γ concentration was suggested that the recipient's immunity would be reinforced.

In relation to administration of activated lymphocytes, CD56 mRNA expression in PBMCs remained unchanged. In comparison to PBMCs, the increase in CD56 expression in activated lymphocytes was obvious. CD56 mRNA expression was reported to be up-regulated in the lymphocytes stimulated by mitogen [4]. Relative quantification of CD56 mRNA expression was likely to have been an indicator of lymphocytes activation.

Among PBMCs, changes in the cell proportions and increase in the IFN- γ production were observed in the dogs used in this study. These results indicated a change in the serum IFN- γ concentration; moreover, the phenotypic assay contributed to recognition of immunoactivation during sequential administration of activated lymphocytes.

In conclusion, activated lymphocytes could be induced using anti-CD3 antibody, hr IL-2 and hr IFN- α in healthy dogs, and sequential administration of these activated lymphocytes reinforced the recipient's immunity in some way. A further study is needed to establish the ideal lymphocyte culture technique and the optimal method for evaluation of its efficacy. Activated lymphocyte therapy, however, was expected to have improved the immunity and the quality of life of immunosuppressed tumor-bearing animals.

REFERENCES

1. Bendelac, A., Rivera, M. N., Park, S. H. and Roark, J. H. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* **15**: 535–562.
2. Bergman, P. J. 2007. Anticancer vaccines. *Vet. Clin. North Am. Small Anim. Pract.* **37**: 1111–1119.
3. Blackwood, L. and Dobson, J. M. 1996. Radiotherapy of oral malignant melanoma in dogs. *J. Am. Vet. Med. Assoc.* **209**: 98–102.
4. Bonkobara, M., Sato, T., Takahashi, N., Kasahara, Y., Yagihara, H., Tamura, K., Isotani, M., Azakami, D., Ono, K. and Washizu, T. 2005. Characterization of cDNA and the genomic sequence encoding canine neural-cell adhesion molecule, CD56/N-CAM. *Vet. Immunol. Immunopathol.* **107**: 171–176.
5. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest. (Suppl.)* **97**: 7.
6. Callan, M. F., Fazou, C., Yang, H., Rostron, T., Poon, K., Hatton, C. and McMichael, A. J. 2000. CD8 (+) T-cell selection, function, and death in the primary immune response *in vivo*. *J. Clin. Invest.* **106**: 1251–1261.
7. Chen, L. K., Tourville, B., Burns, G. F., Bach, F. H., Mathieu-Mahul, D., Sasportes, M. and Bensussan, A. 1986. Interferon: a cytotoxic T lymphocyte differentiation signal. *Eur. J. Immunol.* **16**: 767–770.
8. Craig, L. E. 2001. Cause of death in dogs according to breed: a necropsy survey of five breeds. *J. Am. Anim. Hosp. Assoc.* **37**: 438–443.
9. Ebina, T., Fujimiya, Y., Yamaguchi, T., Ogama, N., Sasaki, H., Isono, N., Suzuki, Y., Katakura, R., Tanaka, K., Nagata, K., Takano, S., Tamura, K., Uno, K. and Kishida, T. 1998. The use of BRM-activated killer cells in adoptive immunotherapy: a pilot study with nine advanced cancer patients. *Biotherapy* **11**: 241–253.
10. Ebina, T., Ogama, N., Shimanuki, H., Kubota, T. and Isono, N. 2001. Effector mechanism and clinical response of BAK (BRM-Activated Killer) immuno-cell therapy for maintaining satisfactory QOL of advanced cancer patients utilizing CD56-positive NIE (neuro-immune-endocrine) cells. *Microbiol. Immunol.* **45**: 403–411.
11. Ebina, T., Ogama, N., Shimanuki, H., Kubota, T. and Isono, N. 2003. Life-prolonging effect of immunocell BAK (BRM-activated killer) therapy for advanced solid cancer patients: prognostic significance of serum immunosuppressive acidic protein levels. *Cancer Immunol. Immunother.* **52**: 555–560.
12. Egawa, K. 2004. Immuno-cell therapy of cancer in Japan. *Anti-cancer Res.* **24**: 3321–3326.
13. Farag, S. S. and Caligiuri, M. A. 2006. Human natural killer cell development and biology. *Blood Rev.* **20**: 123–137.
14. Funk, J., Schmitz, G., Bach, U., Failing, K. and Burkhardt, E. 2003. Influence of different tumour types on natural cytotoxicity (NK cell activity) and mitogen-induced lymphocyte proliferation in isolated blood lymphocytes from 110 dogs with tumours. *Res. Vet. Sci.* **74**: 129–135.
15. Gemlo, B. T., Palladino, M. A. Jr., Jaffe, H. S., Espevik, T. P. and Rayner, A. A. 1988. Circulating cytokines in patients with metastatic cancer treated with recombinant interleukin 2 and lymphokine-activated killer cells. *Cancer Res.* **48**: 5864–5867.
16. Gondolf, C., Burkhardt, E., Failing, K. and Stitz, L. 1996. A new colorimetric method for measuring cell-mediated cytotoxicity in dogs. *Vet. Immunol. Immunopathol.* **55**: 11–22.
17. Grimm, E. A. 1986. Human lymphokine-activated killer cells (LAK cells) as a potential immunotherapeutic modality. *Biochim. Biophys. Acta.* **865**: 267–279.
18. Grimm, E. A., Robb, R. J., Roth, J. A., Neckers, L. M., Lachman, L. B., Wilson, D. J. and Rosenberg, S. A. 1983. Lymphokine-activated killer cell phenomenon. III. Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells. *J. Exp. Med.*

- 158: 1356–1361.
19. Helfand, S. C., Soergel, S. A., Modiano, J. F., Hank, J. A. and Sondel, P. M. 1994. Induction of lymphokine-activated killer (LAK) activity in canine lymphocytes with low dose human recombinant interleukin-2 *in vitro*. *Cancer Biother.* **9**: 237–244.
20. Henkart, P. A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity* **1**: 343–346.
21. Hoshino, Y., Tajima, M., Takagi, S., Osaki, T., Okumura, M. and Fujinaga, T. 2008. Relative quantification of canine CD56 mRNA expression by real-time polymerase chain reaction method in normal tissues and activated lymphocytes. *J. Vet. Med. Sci.* **70**: 309–312.
22. Inoue, K., Ohashi, E., Kadosawa, T., Hong, S.H., Matsunaga, S., Mochizuki, M., Nishimura, R. and Sasaki, N. 2004. Establishment and characterization of four canine melanoma cell lines. *J. Vet. Med. Sci.* **66**: 1437–1440.
23. Ioannides, C. G. and Whiteside, T. L. 1993. T cell recognition of human tumors: implications for molecular immunotherapy of cancer. *Clin. Immunol. Immunopathol.* **66**: 91–106.
24. Itoh, H., Kakuta, T., Kudo, T., Sakonju, I., Hohdatsu, T., Ebina, T. and Takase, K. 2003. Bulk cultures of canine peripheral blood lymphocytes with solid phase anti-CD3 antibody and recombinant interleukin-2 for use in immunotherapy. *J. Vet. Med. Sci.* **65**: 329–333.
25. Kato, M., Watarai, S., Nishikawa, S., Iwasaki, T. and Kodama, H. 2007. A novel culture method of canine peripheral blood lymphocytes with concanavalin A and recombinant human interleukin-2 for adoptive immunotherapy. *J. Vet. Med. Sci.* **69**: 481–486.
26. Kol, R., Friedlander, M., Riklis, E. and Raveh, D. 1983. Separation of human lymphocytes on Ficoll-Paque gradients: stimulation of cells and depletion of a concanavalin-A responsive radioresistant subpopulation. *Radiat. Res.* **95**: 108–115.
27. Kwak, J. Y., Han, M. K., Choi, K. S., Park, I. H., Park, S. Y., Sohn, M. H., Kim, U. H., McGregor, J. R., Samlowski, W. E. and Yim, C. Y. 2000. Cytokines secreted by lymphokine-activated killer cells induce endogenous nitric oxide synthesis and apoptosis in DLD-1 colon cancer cells. *Cell Immunol.* **203**: 84–94.
28. Lanier, L. L., Le, A. M., Civin, C. I., Loken, M. R. and Phillips, J. H. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* **136**: 4480–4486.
29. Lee, P. P., Yee, C., Savage, P. A., Fong, L., Brockstedt, D., Weber, J. S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P. D., Roederer, M. and Davis, M. M. 1999. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat. Med.* **5**: 677–685.
30. Menetrier-Caux, C., Montmain, G., Dieu, M. C., Bain, C., Favrot, M. C., Caux, C. and Blay, J. Y. 1998. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood* **92**: 4778–4791.
31. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**: 2348–2357.
32. Nishimura, T., Nakamura, Y., Takeuchi, Y., Tokuda, Y., Iwasawa, M., Kawasaki, A., Okumura, K. and Habu, S. 1992. Generation propagation, and targeting of human CD4⁺ helper/killer T cells induced by anti-CD3 monoclonal antibody plus recombinant IL-2. An efficient strategy for adoptive tumor immunotherapy. *J. Immunol.* **148**: 285–291.
33. Reinherz, E. L. and Schlossman, S. F. 1980. The differentiation and function of human T lymphocytes. *Cell* **19**: 821–827.
34. Rosenberg, S. A. and Lotze, M. T. 1986. Cancer immunotherapy using interleukin-2 and interleukin-2 activated lymphocytes. *Annu. Rev. Immunol.* **4**: 681–709.
35. Scott, P. and Trinchieri, G. 1995. The role of natural killer cells in host-parasite interactions. *Curr. Opin. Immunol.* **7**: 34–40.
36. Tada, T., Sano, H., Sato, S., Shima, J., Fujiwara, H. and Hamaoka, T. 1990. Immune dysfunction expressed selectively on L3T4⁺ T cells in the tumor-bearing state. *J. Leukoc. Biol.* **47**: 149–157.
37. Takahashi, E., Kuranaga, N., Satoh, K., Habu, Y., Shinomiya, N., Asano, T., Seki, S. and Hayakawa, M. 2006. Induction of CD16⁺ CD56^{bright} NK cells with antitumor cytotoxicity not only from CD16⁺ CD56^{bright} NK cells but also from CD16⁺ CD56^{dim} NK cells. *Scand. J. Immunol.* **65**: 126–138.
38. Takayama, T., Sekine, T., Makuuchi, M., Yamasaki, S., Kosuge, T., Yamamoto, J., Shimada, K., Sakamoto, M., Hirohashi, S., Ohashi, Y. and Kakizoe, T. 2000. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. *Lancet* **356**: 802–807.
39. von Boehmer, H. and Fehling, H. J. 1997. Structure and function of the pre-T cell receptor. *Annu. Rev. Immunol.* **15**: 433–452.
40. Wang, F. S., Liu, M. X., Zhang, B., Shi, M., Lei, Z. Y., Sun, W. B., Du, Q. Y. and Chen, J. M. 2002. Antitumor activities of human autologous cytokine-induced killer (CIK) cells against hepatocellular carcinoma cells *in vitro* and *in vivo*. *World J. Gastroenterol.* **8**: 464–468.
41. Withrow, S. J. and MacEwen, E. G. 1989. Why worry about cancer in pet animals? pp. 1–3. In: *Small Animal Clinical Oncology*, 2nd ed., W. B. Saunders, Philadelphia.
42. Wolfesberger, B., Hoelzl, C., Walter, I., Reider, G. A., Fertl, G., Thalhammer, J. G., Skaliczy, M. and Egerbacher, M. 2006. *In vitro* effects of meloxicam with or without doxorubicin on canine osteosarcoma cells. *J. Vet. Pharmacol. Therap.* **29**: 15–23.