Bulk Cultures of Canine Peripheral Blood Lymphocytes with Solid Phase Anti-CD3 Antibody and Recombinant Interleukin-2 for Use in Immunotherapy

Hiroshi ITOH¹⁾, Tomoko KAKUTA¹⁾, Tetsuya KUDO²⁾, Iwao SAKONJU¹⁾, Tsutomu HOHDATSU¹⁾, Takusaburo EBINA³⁾ and Katsuaki TAKASE¹⁾

¹⁾Department of Veterinary Surgery, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034-8628,

²⁾Hachinohe Animal Hospital, Hachinohe-Shi, Aomori 034 and ³⁾Division of Immunology, Miyagi Cancer Center Research Institute, Natori, Miyagi 981-1293, Japan

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ABSTRACT. Interleukin (IL)-2 can induce large numbers of lymphokine-activated killer cells in peripheral blood lymphocytes (PBL), but IL-2 alone cannot induce proliferation of a large number of canine (c) PBL. We used the solid phase anti-CD3 antibody and soluble recombinant (r) IL-2 in order to establish a large scale culture method for cPBL. The number of lymphocytes seeded (3×10^7) increased to 1×10^9 after incubation for 10 days. The phenotype of cultured cPBL cells (after 2 weeks) showed a CD4⁺ or CD8⁺ predominant cell population. The cultured cell solutions were administered with physiological saline intravenously to each dog. After transfusion of the cultured cells, the cPBL counts, especially the number of CD4⁺, CD8⁺ and CD4⁺CD8⁺ (DN) cells increased significantly in the recipient dogs. Natural killer (NK) cells, $\gamma\delta$ T cells and B cells were considered to be present in the DN cell population. The NK cells and $\gamma\delta$ T cells showed no adverse reaction to the transfusion of the activated cPBL. Therefore, it is necessary to recognize the B cells present in the DN cell population by detecting CD21⁺ cells. In conclusion, the bulk culture system of cPBL with rIL-2 and solid phase anti-CD3 antibody may be useful for the development of novel immunotherapy in dogs.

KEY WORDS: anti-CD3-antibody, bulk culture, canine, interleukin-2, lymphocyte.

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Adoptive immunotherapy has received renewed interest in recent years, and this method has been used in human clinical trials [2–4, 16, 26, 29]. Adoptive immunotherapy for treatment of advanced cancer in humans has been used at many medical institutions in Japan [2, 26, 28, 29]. Several early problems existed in terms of adverse reactions and complex technical procedures, but several improved methods of adoptive immunotherapy have been developed in conjunction with recent advances in the field of immunology. New methods of adoptive immunotherapy with less complex procedures and less risk of adverse reactions have now been established [4].

Studies in animals have evaluated antitumor lymphocyte cultures by using human recombinant (r) interleukin (IL)-2, dog (d) IL-2, and phytohemagglutinin-P (PHA) with canine (c) peripheral blood lymphocytes (PBL) and *in vitro* cytotoxicity with proliferation of lymphocyte activated killer (LAK) cells [8, 9, 11, 14–16, 19]. Results indicate that these methods may be effective as tumor immunotherapy in dogs with cancer [15], but immunotherapy has not been utilized in veterinary practice because larger scale PBL culture techniques have not been established. Because of their shorter life spans, animals with cancer tend to die earlier than humans due to complications. Therefore, the use of immunotherapy in animals may represent a significant advance in terms of prolonging and improving the quality of life.

Large scale cultures of human (h) PBL currently combine the use of anti-CD3 antibody and rIL-2 [2]. Several studies have shown that this combination induces larger quantities of LAK cells as compared to cultures with rIL-2 alone [8, 18, 28]. The biological response modifiers-activated killer

(BAK) culture was shown to proliferate on treatment with IL-2 and solid-phased anti-CD3 antibody [2–4]. This resulted in killing of tumor cells but no impairment of normal mononuclear cells [4].

The present study was designed to establish the bulk culture system of cPBL in the dog with anti-CD3 antibody and rIL-2 for its clinical use as immunotherapy.

MATERIALS AND METHODS

Experimental animals: The study was carried out in 4 dogs, including two beagle dogs (1 male and 1 female) and two mixed breed dogs (1 male and 1 female). General physical examination and laboratory testing of the dogs revealed no abnormalities. The study was conducted in accordance with the guidelines of the Kitasato University Committee on Animal Care and Use.

Preparation of anti-CD3 antibody flasks: The bottoms of cell culture flasks (Cellstar®; Greiner, Frickenhausen, Germany) were coated with a mixture of 0.5 ml of anti-CD3 antibody (mouse anti-canine CD3, 50 μ g/ml; Dainippon Pharmaceutical, Tokyo, Japan) in 4.5 ml of physiological saline solution (final concentration, 5 μ g/ml). These were allowed to stand at room temperature for 24 hr [3,4].

Preparation of culture medium: The cell culture medium used as a complete medium (CM) (CM; IL-2 700 relative unit (RU)/ml, 10% fetal bovine serum) which comprised a 2:7:1 ratio of RPMI-1640+7 (h) T cell liquid medium, L-glutamine (L-Glu), and NaHCO₃; Nikken Seibutsu Igaku Kenkyujo, Kyoto, Japan), RPMI-1640+9 (hT cell liquid medium, L-Glu, NaHCO₃, and IL-2 1,000 RU/ml; Nikken

Seibutsu Igaku Kenkyujo) and inactivated fetal bovine serum (FBS).

Separation and culture of cPBL: Heparinized peripheral blood samples from the dogs were collected and diluted to a 1:1 ratio with physiological saline. The lymphocytes were then separated with Ficoll (Lymphoprep, Nycomed Pharma, Oslo, Norway) [12]. The canine PBL were adjusted to a final concentration of 3×10^7 cells/10 ml of PBL suspension. The PBL suspension was incubated in an anti-CD3 antibody coated flask at 37°C for 14 days in a 5% CO₂ incubator. On days 1 and 4 after incubation, 7 ml and 13 ml, respectively, of CM was added to the culture solution in the flask. On day 5, 10 ml of the cell culture solution was dispensed into each of 2 flasks. In addition, 20 ml of CM was added to each of the 3 culture flasks. On day 6, the culture solutions from the 3 flasks were collected in one culture bag (RPMI-1640 containing 2% FBS and IL-2 175 RU/ml). On day 8, all of the medium from a fresh culture bag was infused into the culture bag through a connecting tube. After being mixed, one half of the medium was returned to the bag (total of 2 bags). On day 11, the same procedure was followed to prepare a total of 4 bags. The solutions from each bag were centrifuged (3,000 rpm, 20°C, 9 min), and the supernatant was discarded. Next, 15 ml of RPMI-1640+9 and 10 µl (1,000 U/ml) of interferon (IFN) ω (Intercat, 10 MU/ml; Toray Industries, Tokyo, Japan) were added to the harvested cells. After being mixed well, the cells were incubated in CO₂ for 15 min at 37°C. After incubation, the cells were washed twice with 15 ml of washing solution, resuspended in 100 ml of a final solution (3% canine albumin), and then infused into a blood transfusion bag. The cultured cell solutions were transfused over about 1 hr (after infusion of physiological saline) into the radial vein of each dog. The number of cells was counted in the cell suspension by means of a Burker-Turk counter. The cell survival rate was also determined.

Phenotype assay: 1×10^6 PBL cells were added to each of two 5 ml polystyrene round tubes. These were centrifuged (1,700 rpm, 4°C, 3 min), and the supernatants were discarded. Ten µl of fluorescent isothiocyanate (FITC) labeled anti-IgG antibody (goat anti-mouse IgG. FITC-rat absorbed; Serotec, Dainippon Pharmaceutical) was added to one of the tubes as the negative control. Ten (1 each of fluorescent isothiocyanate (FITC) labeled anti-CD4⁺ antibody (rat anti-canine CD4⁺; FITC; Serotec, Dainippon Pharmaceutical) and R-phycoerthrin(R-PE) labeled anti-CD8⁺ antibody (rat anti-canine CD8⁺; R-PE; Serotec, Dainippon Pharmaceutical) were added to the sediment in the other tube. The samples were chilled and reacted for 30 min, then washed 3 times with cold PBS containing 0.1% Na₂N₃. The samples were resuspended in 300 µl of sheath solution (Isoflow: Beckman Coulter, Kanagawa, Japan). Phenotyping of the lymphocytes was done by flow cytometric analysis (FACScan: Becton Dickinson, San Jose, CA, U.S.A.) with analysis software (Immunocytometry Systems: Becton Dickinson).

Sterility testing and endotoxin assay: Testing for isola-

tion of microbes in the culture solutions was done with agar and Sabouraud's agar media. Assay for endotoxins in the culture solutions was done with endotoxin assay kits (Toxicolor® Et-1 Set and Fungitec® G Test TE: Seikagaku-kogyo, Tokyo, Japan).

RESULTS

Canine PBL cultures: The cells cultured in the solid-phase anti-CD3 antibody flasks showed good proliferation 4 to 10 days after cultivation. Maximum cell counts were achieved by day 12, except for the cultured cells from one dog (No. 3). The PBL from dog No. 3 showed continued gradual growth from day 10 to day 14. The cell counts increased approximately 60–106-fold as compared to that before cultivation (3×10^7 to $1.4\text{--}3.2 \times 10^9$) (Fig. 1). The cultured cells from cPBL were treated with 10 µl of IFN-ω for 15 min on the last day of incubation in order to activate natural killer (NK) cells, but the enhancement of NK was not confirmed.

Cell survival rate: The survival rates of cultured cells for 3 dogs (excluding one dog) had gradually decreased by culture day 12 but were generally 80% or higher. The cell survival rates on day 14 from Dogs 1, 2, 3 and 4 were 43.1%, 45.1%, 77% and 57.0%, respectively.

Fig. 1. Changes in proliferation of canine blood lymphocytes induced with anti-CD3 antibody and rIL-2. The cells proliferated in response to solid-phase anti-CD3 antibody and rIL2 of peripheral blood lymphocyte in dogs (—■—=No 1, ...●...=No 2, —▲—=No 3, ---□---=No 4). The number of cells increased to 1×10^9 by day 10 from the start of incubation, but the subsequent growth of cells was slower. Maximum cell counts were achieved by day.

Table 1. Total numbers of lymphocyte and phenotypes of lymphocytes stimulated with anti-CD3 antibody plus recombinant interleukin-2 for 2 weeks

No		Number ^{a)} of cPBL and culture cell	Phenotype of lymphocyte (%)			
			CD4	CD8	DP	DN
1	Before	3.0×10^7	40.5	26.7	1.0	31.8
	After 2 weeks	1.4×10^9	59.0	14.5	20.5	6.0
2	Before	3.0×10^7	44.2	25.7	0.4	29.7
	After 2 weeks	1.4×10^9	59.0	18.0	17.0	6.0
3	Before	3.0×10^7	47.0	28.5	1.7	22.9
	After 2 weeks	1.8×10^9	24.0	54.0	4.0	18.0
4	Before	3.0×10^7	43.2	31.6	2.3	22.9
	After 2 weeks	1.6×10^9	5.0	75.8	2.0	17.0

DP:CD4⁺CD8⁺ double positive. DN:CD4⁺CD8⁻ double negative.

a) Number of the peripheral blood lymphocytes (cPBL) before and after the transfusion of cPBL stimulated with rIL-2 and anti CD3 antibody.

T cell classification of cultured cells and after administration of cultured cells: Analysis of the T cell surface markers of the cultured cells from cPBL showed the proportion of CD4⁺ (Dog1 and 2) and CD8⁺ (Dog3 and 4) cells to be increased after 14 days of culture as compared to cPBL before administration of cultured cells to the experimental dogs. The CD4⁺CD8⁺ double positive (DP) cells increased in Dogs 1 and 2 (20.5% and 17%), but did not change in Dogs 3 and 4. The CD4⁺CD8⁻ double negative (DN) cells in Dogs 1 and 2 decreased (6.0% and 6.0%) as compared to cPBL before the administration of cultured cells (31.8% and 29.7%), but slightly decreased in Dogs 3 and 4 (Table 1).

T cell surface marker analysis of cPBL before transfusion of the cultured cells showed that the CD4⁺ cells accounted for about one half of the cells. The number of DP cells was low in the dogs. The number of CD4⁺, CD8⁺ and DN cells increased after transfusion of the cultured cells. The number of DN cells had significantly increased at 2 weeks after transfusion of the cultured cells (Table 2).

General hematologic and biochemical laboratory studies: General biochemical laboratory studies done before and 1 and 2 weeks after treatment revealed no abnormal findings in any dog. But, the cPBL administered increased the number of cells in comparison with the cPBL number before the administration, and the number of lymphocytes continued for remained unchanged about 2 weeks (Table 2).

Examination of the stability and endotoxin concentration: Testing was performed for microbial contamination and the endotoxin concentrations in the culture solutions to confirm the safety of the cultured cells for transfusion. The endotoxin concentrations of the culture solutions were within normal limits (2.95–8.92 pg/ml). Smear cultures on agar and Sabouraud's agar media revealed no colony formation of any microorganisms.

DISCUSSION

Like hrIL-2, cIL-2 has also been reported to activate and

Table 2. Number of canine peripheral blood lymphocytes and phenotype of canine peripheral blood lymphocytes before and after transfusion of the culture cells to the dogs

No		cPBL	CD4	CD8	DP	DN
1	Before	2,940	1,141	54	27	898
	After 1 week	5,292	2,065	1,457	95	1,674
	After 2 weeks	4,234	1,727	1,095	35	1,376
2	Before	3,650	1,613	939	14	1,084
	After 1 week	5,811	2,702	1,472	62	1,575
	After 2 weeks	5,771	2,258	1,368	21	2,124
3	Before	4,956	2,328	1,411	84	1,133
	After 1 week	4,628	2,184	1,123	50	1,271
	After 2 weeks	5,950	2,328	1,731	43	1,846
4	Before	2,943	1,270	929	69	675
	After 1 week	4,235	1,556	929	18	1,722
	After 2 weeks	6,916	2,716	1,478	23	2,699

Each value is the number of cPBL (/μl) and phenotypes of cPBL (/μl) in the dogs. DP:CD4⁺CD8⁺ double positive. DN:CD4⁺CD8⁻ double negative.

stimulate the production of T cells [2, 11, 14, 16, 19]. The T cells of cPBL were cultured in a flask with solid-phased canine anti-CD3 antibody and activated with rIL-2. The cultured cell count gradually increased from 3×10^7 to 1×10^9 cells by day 10. In a previous study in mice, 10^6 – 10^8 lymphocytes were found to be necessary for tumor size reduction [7]. Based on data from that mouse model, we estimated that 10^8 – 10^{10} cells would be required for a similar effect in dogs. The cultured cell count of 10^9 in the present study is considered to be a sufficient number for adoptive immunotherapy of tumors. The solid-phase canine anti-CD3 antibody used in this study, as in humans, probably stimulates T cells and leads to expression of IL-2R [18]. Activation by rIL-2 also produces gradual cell proliferation [16]. Furthermore, the solid-phase anti-CD3 antibody induces the production of LAK cells with so-called tumor selective cytotoxicity but no harmful effects on other autologous cells [1, 2]. A longer duration of the solid-phase anti-CD3 antibody effect also increases the cell growth rate [5, 20, 21, 23, 26, 28], so that anti-CD3 antibody is thought to be an essential factor for immunotherapy in dogs.

It has been reported that the NK cells were reactivated by 500 U/ml of IFN- α and 1,000 U/ml of IL-2 for 1 hr [2]. In this study, for utilizing activated NK cells, 1,000 U/ml of IFN- α was added to the harvested cells, but no enhancement of NK cell or cultured cells activities was confirmed.

In the cPBL after cultivation for 2 weeks, CD4⁺ or CD8⁺ cells were predominant populations. CD4⁺ T cells and CD8⁺ T cells are known to primarily have a helper effect and a cytotoxic effect on tumors, respectively [6, 13, 23–25]. After transfusion of the cultured cells, T cell surface marker analysis generally showed an increase in CD4⁺, CD8⁺ cells and DN cells. Cultured cells with predominant proliferation of CD8⁺ cells and DN cells would be expected to have an antitumor effect [4, 10, 25, 28], but the increase in the B cell which is one of the components of the DN cell is also considered. Therefore, it is necessary to recognize the B cell on the cPBL and cultured cells by detecting the CD21⁺ cell.

Cultures of human cells with solid-phased anti-CD3 antibody and IL-2 primarily show proliferation of CD4⁺, CD45R⁻ helper-inducer cells and CD8⁺, CD28⁺ cytotoxic T lymphocytes [5]. Based on our findings, this method with canine cells probably yields similar cellular proliferation responses as with human cells.

The peripheral lymphocyte counts were increased in each of the dogs at 1 week after transfusion of the cultured cells. Higher counts were also maintained by 2 weeks after transfusion in 4 of the experimental dogs. These findings are in agreement with results in humans who have received transfusions of hPBL that have been proliferated with anti-CD3 antibody [17]. The findings also suggest that the transfused lymphocytes indeed play a role in *in vivo* immune responses.

Observation of the cultured cell survival rates showed a decrease in the number of cells and inhibition of cell proliferation starting on day 12 after incubation. In a study by Mizuno *et al.* [15] on the long-term cultures of cPBL with PHA and rIL-2, frequent cell stimulation with PHA was necessary to maintain the cell count and survival rate. The reason why cell counts could not be maintained by rIL-2 alone was thought to be due to relatively early depletion of rIL-2 receptors. The proliferating cPBLs themselves probably utilize and deplete the rIL-2. Therefore, when using the method described in this paper for adoptive immunotherapy, the cell counts, cell survival rates, and quantity of culture solution must continuously be monitored in order to determine the most appropriate time for transfusion of the cultured cells.

Successful immunotherapy depends on many factors, including: whether a sufficiently large quantity of antitumor cells can be cultured; the antitumor activity of each cell unit; tumor cell aggregation with the cultured cells; specific binding and cytotoxic activity against the tumor cells; the *in vivo* half-life of the cells; and the extent to which antitumor activity is not readily decreased by immunosuppression in a given cancer patient [22, 27]. Consequently, further research will be necessary to more accurately determine the cytotoxic effects of immunotherapy utilizing by cPBL whose proliferation is stimulated by solid-phased anti-CD3 antibody and rIL-2.

Safety evaluation included microbial cultures and endotoxin assays of the culture solutions. The lack of colony formation on agar and Sabouraud's agar media confirmed the absence of any microbial contamination. Assay of endotoxin concentrations in the culture solutions for each culture bag showed values below the limits for rabbit pyrogen. Because the cultured cells were washed immediately before transfusion, these concentrations were probably even lower when the cells were actually transfused. There was no clinical abnormality or development of an adverse reaction in any dog that was transfused with these cells.

In conclusion, we established a method for large scale culture of cPBL with solid-phased anti-CD3 antibody and rIL-2. Further *in vitro* cytotoxicity testing and clinical studies should be carried out to enable the use of this type of

adoptive immunotherapy in clinical veterinary practice for treatment of disorders such as advanced cancer in dogs.

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