A Novel Culture Method of Canine Peripheral Blood Lymphocytes with Concanavalin A and Recombinant Human Interleukin-2 for Adoptive Immunotherapy

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ABSTRACT. This study was designed to develop a novel culture method for the efficient proliferation of canine peripheral blood lymphocytes (cPBL) for adoptive immunotherapy. When cPBL were cultured in the presence of concanavalin A (Con A), proliferation of cPBL was induced and expression of interleukin-2 receptor (IL-2R) which enables to respond to exogenously added IL-2 was upregulated. And then, when cPBL were cultured with recombinant human interleukin-2 (rhIL-2) in addition to Con A, proliferation was accelerated and increased to about 10-fold after 1 week. The phenotypic analysis showed that the main population of the cultured cPBL was consisted of CD8+ positive lymphocytes. Among them, CD4+CD8+ double positive (DP) lymphocytes had significantly increased, and the ratio of CD4+ single positive (SP) lymphocytes to CD8+ SP lymphocytes (CD4+SP/CD8+SP) was decreased as compared to before culturing.

To evaluate the cytotoxic activity of cPBL cultured with Con A and rhIL-2, furthermore, cytotoxic assay was carried out against xenogeneic melanoma cell line (MeWo), which resulted in MHC-unrestricted cytokilling. These results suggest that the culture method of cPBL by the use of Con A and rhIL-2 may be useful for generating lymphokine activated killer cells, and also this may be beneficial for adoptive immunotherapy of tumor-bear dogs.

KEY WORDS: canine, concanavalin A, interleukin-2, LAK cell, lymphocyte.

Lymphokine activated killer (LAK) cells is known to posses potent tumoricidal effect [9, 10], thus these cells have been intently investigated as effector killer cells for adoptive immunotherapy, which is expected as a less side effect cancer treatment [24, 33]. Actually, this method has been proved to be effective in human clinical trial, especially in treatment of advanced cancer [3, 4]. Also in companion animals, on the other hand, neoplastic diseases have increased as their life-style is changing [20]. Thus far, there have been a few reports that immunotherapy may be an effective method for tumor-bearing dogs [8, 14]. So, adoptive immunotherapy has been widely noticed in veterinary practice as a newly therapeutic strategy for animals with cancer.

In general, LAK cells for adoptive immunotherapy are generated from peripheral blood lymphocytes (PBL) cultured with anti-CD3 antibody that upregulates IL-2 receptor (R) expression on lymphocytes [31, 32] and rhIL-2 to prepare substantial number of killer cells [16, 31]. In previous study, it has been reported that rhIL-2 induces the proliferation of PBL from several animals species including canine [7, 11, 29]. The proliferation of canine lymphocytes by concanavalin A (Con A), phytohemagglutinin or pokeweed mitogen, which are T cell mitogen in human, has also demonstrated [19]. In addition, it has been reported that Con A induces IL-2R expression on human lymphocytes [18] like anti-CD3 antibody. Thus, it is likely that canine LAK cells would be generated by culturing canine PBL (cPBL) with Con A and rhIL-2. However, induction of canine LAK cells from cPBL by treatment with Con A and rhIL-2 has not yet been presented. In this investigation, we show that canine LAK cells can be generated from PBL by the use of Con A and rhIL-2. We believe that this culture system of cPBL with Con A and rhIL-2 is useful for generation of LAK cells for immunotherapy of canine tumors.

MATERIALS AND METHODS

Experimental animal: Three female beagle dogs, 1–6 year of age were used in experiments. They were housed indoors and maintained for study in accordance with the guidelines of the Osaka Prefecture University Committee on animal care and use.

PBL isolation and cell culture: Heparinized peripheral blood from healthy dogs was diluted with an equal volume of phosphate buffer saline (PBS), and then cPBL were purified by lympholyte-mammal (Cedarlane Laboratories Ltd., Ontario, Canada) according to its instruction. Purified cPBL were washed three times with PBS, and suspended with complete medium (RPMI1640 supplemented with 10% fetal calf serum (FCS), 2% pooled canine serum (CS), 0.03% L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin). Cell suspension was concentrated at 5 x 10^6/ml, then added to 6-well culture plates by 2 ml. Cells were
cultured with Con A (1, 5, 10, and 15 µg/ml) (Elastin Products Company, Inc., Missouri, U.S.A.) at 37°C in a humidified 5% CO₂ atmosphere. Furthermore, to study the effect of rhIL-2 on Con A-stimulated cPBL, cPBL stimulated with Con A (5 µg/ml) were cultured with or without rhIL-2 (0, 250, and 750 U/ml) (PeproTech EC Ltd., London, UK).

**Flow cytometry**: Cultured cPBL were harvested, and washed three times with PBS, and concentrated at 1 × 10⁶/100 µl with PBS/BSA solution (PBS containing 20 mM glucose, 0.1% bovine serum albumin, 0.1% NaN₃, and 2% FCS, pH 7.4). For IL-2Rα expression analysis, cells were reacted with mouse anti-human IL-2 receptor antibody (American Research Products, Inc., Belmont, U.S.A.), followed by FITC-conjugated goat anti-mouse IgG (H+L) (Zymed Laboratories, Inc., San Francisco, CA, U.S.A.). For phenotypic analysis, on the other hands, cells were reacted with FITC-conjugated rat anti-canine CD4 antibody (Serotec Ltd., Oxford, UK) and PE-conjugated rat anti-canine CD8 antibody (Serotec Ltd.). As isotypic control, FITC-conjugated rat IgG2a (Cedarlane Laboratories Ltd.) and PE-conjugated rat IgG 1κ (Cedarlane Laboratories Ltd.) was used. After 3 times wash with PBS, cells were suspended in 1 ml of PBS/BSA solution, and analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.) with CELLQuest software (Becton Dickinson). Forward and side scatter futures were used to select the lymphocyte subpopulation for analysis.

**Cytotoxicity assay**: Cytotoxic activity of cultured cPBL was assessed using human melanoma cell line (MeWo) as the target cell. For the effector cells preparation, cPBL were cultured with Con A (5 µg/ml) and rhIL-2 (750 U/ml) for 7 days, then non-adherent cells were harvested, and washed three times with PBS. Freshly isolated cPBL were used as the control of effector cells. The assay was performed as followed in triplicate. Briefly MeWo was suspended at 1.5 × 10⁵/400 µl with complete medium and added to 24 well plate. Following 4 hr incubation at 37°C in a humidified 5% CO₂ atmosphere, effector cells were added to MeWo culture at an effector to target ratio of 33:1, then the plate was centrifuged at 50 × g for 10 min at room temperature. After 16 hr incubation, adherent MeWo was harvested and the number of viable cells were counted.

**RT-PCR**: For granzyme B (GrB) mRNA detection, total RNA was isolated from cPBL cultured with Con A (5 µg/ml) and rhIL-2 (750 U/ml) for 7 days by Trizol reagent (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) according to its instruction, and stored at –80°C until use. Non-stimulated cPBL cultured for 4 days were used as the source of negative control. For IL-2Rα mRNA detection, cPBL were cultured with only Con A for 3 days. Reverse transcription was performed by using TaKaRa RNA PCR Kit (TAKARA BIO INC., Shiga, Japan). Polymerase chain reactions of canine GrB, canine β-actin and canine IL-2Rα were performed as described previously [2, 15].

**RESULTS**

**Proliferative effect of Con A on cPBL**: To determine the optimal concentration of Con A for cPBL proliferation, PBL from dogs were cultured with various amounts of Con A (1 to 15 µg/ml). As shown in Fig. 1, Con A showed no dose-dependent proliferative effect. Five µg/ml of Con A induced higher cPBL proliferation than other concentrations tested, and the number of cPBL increased approximately 3 times at 8 days as compared to that of pre-cultured cPBL. The data obtained here indicates that the optimal concentration of Con A for cPBL culture is 5 µg/ml.

**IL-2R expression on cPBL stimulated with Con A**: The expression of IL-2R on cPBL after Con A stimulation was studied by flow cytometric analysis. As shown in Fig. 2a, IL-2Rα positive cells represented 13.6% among the resting cPBL. And following Con A stimulation, IL-2Rα expres-
IL-2Rα expression was upregulated, reaching a maximum of 22.8% of IL-2Rα positive cells on day 5. To confirm this observation at molecular level, IL-2Rα specific PCR was performed using RNA sample extracted from Con A-stimulated cPBL (Fig. 2b). In resting cPBL, no or very low level of IL-2Rα mRNA was expressed. After stimulation with Con A, however, PCR product representing IL-2Rα resulted in higher intensity band. This result further confirmed the finding that Con A upregulates IL-2Rα expression on cPBL as indicated by flow cytometric analysis.

Stimulation of cPBL in the presence of both Con A and rhIL-2: To examine the effect of rhIL-2 to Con A-stimulated cPBL, cPBL were cultured with Con A (5 µg/ml) and various concentrations of rhIL-2. As shown in Fig. 3, the proliferative effect of Con A on cPBL was accelerated in the presence of rhIL-2 dose-dependently. The addition of 750 U/ml of rhIL-2 resulted in about 10-fold growth of cPBL during 8 days culturing. This result suggests that the maximal proliferative response of cPBL are observed when cPBL were stimulated with 5 µg/ml of Con A and 750 U/ml of rhIL-2.

Phenotypic analysis of cPBL cultured with Con A and rhIL-2: To clarify cPBL cellular phenotypes after cultivation with Con A and rhIL-2, flow cytometric analysis was performed. Results are shown in Table. 1. In resting cPBL, CD4+ cells showed an overall predominance over CD8+ cells, as expressed by CD4/CD8 ratios more than 1.5. After 7 days culturing with Con A and rhIL-2, however, CD8+ cells were predominant in cPBL cultures, as expressed by CD4/CD8 ratios less than 0.3. In addition, the number of CD4+CD8+ double positive (DP) cells increased with culture, while the number of CD4–CD8– double negative (DN) cells decreased.

Analysis of killer activity of cPBL cultured with Con A

Fig. 2. Analysis of IL-2Rα expression on Con A-stimulated cPBL. cPBL were cultured with Con A (5 µg/ml) for 5 days. IL-2Rα positive cells in non-stimulated cPBL (dot line) or Con A-stimulated cPBL (solid line) was analyzed by flow cytometry (a). The values represent the percentage of IL-2Rα positive cells. Gene expression of IL-2Rα in Con A-stimulated cPBL for 3 days culture and non-stimulated cPBL was analyzed by RT-PCR method (b). Beta-actin was used as the internal control.

Fig. 3. Effect of rhIL-2 on the Con A-stimulated cPBL. cPBL stimulated with Con A (5 µg/ml) were cultured with or without rhIL-2 (0, 250, and 750 U/ml). Data are expressed as mean ± SE of three experiments in triplicate. * p<0.05 as compared with the value of 250 U/ml of rhIL-2. ** p<0.05 as compared with the value of 0 U/ml of rhIL-2.
To reveal whether cPBL cultured with Con A and rhIL-2 exhibit killer activity in MHC-unrestricted manner like LAK cells, cytotoxic assay was carried out using xenogeneic MeWo cell line as the target. As shown in Fig. 4a, cPBL stimulated with Con A and rhIL-2 were significantly more cytotoxic against MeWo cells than resting cPBL under the same conditions. To reveal the killer mechanism of the stimulated cPBL, moreover, expression of GrB, which is a cytolytic enzyme, was examined by RT-PCR. As shown in Fig. 4b, GrB mRNA was detected more prominently in stimulated cPBL than resting cPBL. Taken together, these findings suggest that cPBL cultured with Con A and rhIL-2 have MHC-unrestricted cytotoxic activity mediated by at least GrB activity.

DISCUSSION

Adoptive immunotherapy has been used for treatment of advanced cancer in human [3, 4]. In the field of veterinary medicine, it has been also received attention as a potential therapeutic strategy for animal cancer. LAK cells have been generally prepared by using anti-CD3 antibody and rhIL-2 [16, 31]. In the present study, we used Con A and rhIL-2 for generating of canine LAK cells. Con A was able to induce proliferation of cPBL. However, proliferative effect of Con A on cPBL was not dose-dependent, showing a maximum proliferation at the concentration of 5 µg/ml (Fig. 1). This result is consistent with previous report about mouse and human lymphocytes [34]. Possible explanation of low proliferative effect at high dose of Con A is binding of Con A induced changes on or in the lymphocytes membrane that inhibit free diffusion of receptors [35]. These observations lead us to the idea that more than 5 µg/ml of Con A is inhibitory for cPBL proliferation, and the optimal concentration for cPBL culture is 5 µg/ml.

For efficient lymphocytes proliferation, IL-2R expression is indispensable because it enables to utilize rhIL-2 added exogenously [31]. IL-2R consists of three subunits, such as IL-2Rα, β, and γ. IL-2R/β and γ are constitutively expressed at the cell surface of resting lymphocytes, while IL-2Rα is only appeared on activated lymphocytes [13]. Thus, the responsiveness of lymphocytes to rhIL-2 would depend on

![Graph](image)

**Fig. 4.** Cytotoxic activity of cPBL cultured with Con A and rhIL-2. Human melanoma cell line (MeWo) was used as the target. No. 1, MeWo cells; No. 2, MeWo cells mixed with freshly isolated cPBL; No. 3, MeWo cells mixed with cPBL cultured with Con A (5 µg/ml) and rhIL-2 (750 U/ml). Data are expressed as mean ± SE of the three experiments in triplicate (a). Gene expression of GrB in cPBL cultured with Con A (5 µg/ml) and rhIL-2 (750 U/ml) and non-stimulated cPBL was analyzed by RT-PCR method. Beta-actin was used as the internal control. *p<0.05.
the expression of IL-2Rα. In this study, we showed that Con A upregulated IL-2Rα expression on cPBL (Fig. 2). This finding suggests that rhIL-2 would be able to induce higher proliferation of cPBL stimulated with Con A. Indeed, the growth of Con A-stimulated cPBL was accelerated in the presence of rhIL-2 with dose-response correlation (Fig. 3). For clinical use of adoptive immunotherapy in dogs, a large number of LAK cells would be required. It has been reported that more than $1 \times 10^8$ LAK cells would be necessary for immunotherapy in dogs [14]. In this study, we use small scale cPBL culture for preparation of LAK cells. However, we have already established a method for large scale culture of cPBL with Con A and rhIL-2. By this method, the number of lymphocytes was heightened by about 100–150-fold and more than $1 \times 10^8$ LAK cells could be prepared (manuscript in preparation).

In flow cytometric analysis, it was found that the main population of cPBL cultures with ConA and rhIL-2 for 7 days consisted of CD8+ T cells, and that DN lymphocytes was very small population (Table 1). It has been reported that LAK activity is mediated by NK cells [6]. NK cells are also known as MHC-unrestricted cytotoxic lymphocytes [23]. Thus, cytotoxic activity of cPBL stimulated with Con A and rhIL-2 (Fig. 4a) may not be contributed to NK cells which is present in the DN lymphocytes, but NK T cells of which subpopulation with CD8+ phenotype was described in human [30]. However, further study is required to verify whether the cytotoxicity exerted by cPBL cultured with Con A and rhIL-2 is attributed to the NKT cells. Moreover, the number of CD4+CD8+ DP T cells significantly increased with culture using Con A and rhIL-2, while CD4+ SP T cells decreased as compared to the resting cPBL. In rat T cells, previously it was shown that CD4+ T cells co-expressed CD8 molecule upon activation [17], which might explain the above observation.

Peripheral appearance of the CD4+CD8+ DP T cells in Primates and other mammals has been described in some studies [12, 25, 26]. The precise role of these T cells is not clearly understood. However, it has been demonstrated that CD4+CD8+ DP T cells in cynomolgus monkeys showed both helper and cytotoxic activities, and their cytotoxic activity was mainly mediated by perforin and granzyme [25]. Furthermore, it is well known that for efficient immune response against tumor cells, not only cytotoxic T cells but helper T cells are required [21]. The appearance of CD4+CD8+ DP T cells is also known to be associated with neoplastic diseases [12]. From these evidences, it is likely that cPBL cultured with ConA and rhIL-2 modulate immune response and enhance anti-cancer immunity when the cultured-cPBL were transfused. However, additional studies will be necessary to clarify the precise character of increasing CD8+ T cells and the functional feature of CD4+CD8+ DP T cells in the cultured-cPBL.

LAK cells exert their killer activity through perforin/granzyme pathway [28] in MHC-unrestricted manner [6, 27] like NK cells. This means that they lyse MHC-negative or -mismatched target cells without any damage to normal self-tissue. In the present study, it was showed that cPBL stimulated with Con A and rhIL-2 have MHC-unrestricted cytotoxic activity (Fig. 4a). In addition, an acceleration of GrB mRNA expression was also confirmed in the stimulated cPBL (Fig. 4b). Furthermore, previous study showed that human lymphocytes cultured with Con A have specific cytotoxicity toward cancer cells [1]. From these observations, it seems reasonable to suppose that the cytotoxic activity of cPBL stimulated with Con A and rhIL-2 is comparable to LAK activity that is induced by anti-CD3 antibody and rhIL-2. Thus, the stimulated cPBL might be effective as killer cells for adoptive immunotherapy. However, it is necessary to clarify whether stimulated cPBL have no effect on normal tissues.

In the present study, finally, we showed a novel culture method that can generate LAK cells from cPBL by the use of ConA and rhIL-2. Adoptive immunotherapy is gaining increased interest as potential therapy for dogs with cancer. For clinical use of cPBL stimulated with Con A and rhIL-2 in cancer immunotherapy for dogs, however, it is necessary to test the cytotoxic activity of cPBL after transfusion of the cultured cells, and also to assess the clinical abnormality and the adverse events in dogs transfused with the cultured cPBL. Further studies are in progress to verify these points.

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