Characterization of Dog Interleukin-2 Activity

Shinya MIZUNO, Toru FUJINAGA, and Mitsuyoshi HAGIO

Department of Veterinary Surgery, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

ABSTRACT. Proliferative activity of murine interleukin-2 (IL-2) dependent T cells (CTLL-2) was detected in the culture supernatant of canine peripheral blood lymphocytes (PBL) stimulated with phytohemagglutinin-P (PHA-P), and was defined as dog IL-2. The highest production of IL-2 was obtained under the conditions in which a PBL population of 2×10^6 cells/m*l* was stimulated with PHA-P at a concentration of 10 μg/m*l* at 38°C for 48 hr. Dog IL-2 activity was significantly inhibited by heating at 65°C, acidification under pH 4, alkalification over pH 10, and trypsin exposure. A peak of dog IL-2 activity was detected in the fraction with a molecular weight of approximately 31,000 by gel filtration. Long-term culture of canine lymphocytes was successful over 10 passages by using dog IL-2 with PHA-P-stimulation every 3 passages. The cultured cells mostly consisted of small- and medium-sized lymphocytes. These cells reacted to anti-dog thymocyte rabbit serum and anti-dog Thy-1 mouse monoclonal antibody. These cells were therefore considered to originate in T-lineage lymphocytes. Cytostasis of PBL from intact dogs reacting to canine transmissible venereal sarcoma cells was increased significantly when PBL was cultured for more than 30 days with homologous IL-2.—KEY WORDS: canine, interleukin-2, lymphocyte, lymphokine-activated killer cell, tumor.

——————————————————————————————————— *J. Vet. Med. Sci.* 55(6): 925–930, 1993

A humoral factor which propagates bone marrow cells was detected in conditioned medium of human peripheral blood lymphocytes (PBL) stimulated with phytohemagglutinin-P (PHA-P) [22]. It was later named T cell growth factor (TCGF) [1], and was finally called interleukin-2 (IL-2). IL-2 could play important roles in the differentiation of cytotoxic T lymphocytes (CTL) [25, 30], as well as in the induction and proliferation of natural killer (NK) cells and lymphokine-activated killer (LAK) cells [12, 19, 28, 29]. Autologous LAK cells have been administrated clinically as cancer immunotherapy, "LAK therapy" [15, 26].

In man and some animals, several reports of IL-2 production and its properties have been published [1, 7, 8, 11, 27]. However, to our knowledge, there are very few accounts in the literature on the dog IL-2 [3, 5].

In the present study, a growth factor of murine IL-2 dependent cloned T cells (CTLL-2) was detected in supernatants of canine PBL stimulated with PHA-P. The factor was defined as dog IL-2 and the optimal conditions for its production were determined. Some physicochemical properties of dog IL-2 were also examined. In addition, the effect of canine lymphocytes cultured with homologous IL-2 on tumor cells was investigated.

MATERIALS AND METHODS

Animals: Six clinically healthy beagle dogs aged 4 to 7 years weighing 7 to 12 kg were used. Three of them were later implanted with canine transmissible venereal sarcoma (CTVS) which had been maintained by allogenic serial transfer in our laboratory [18]. After spontaneous regression of the tumor, these were called CTVS-sensitized dogs.

Culture medium and isolation of PBL: RPMI-1640 (Gibco Lab., New York, U.S.A.) containing 10 mM HEPES, penicillin-G (100 U/m*l*) and streptomycin (100 μg/m*l*) was used as a complete medium (CM). Canine PBL was isolated from heparinized peripheral venous blood as previously described [21].

IL-2 preparation: Canine PBL were adjusted to 2×10^6 cells/m*l* in CM containing 2% fetal calf serum (FCS, Hazleton Biologics Inc., Lenexa, U.S.A.) and 10 ng/m*l* phorbol 12-myristate 13-acetate (Sigma Chemical, ST. Louis, U.S.A.). IL-2 production from PBL stimulated with PHA-P (Difco Lab., Detroit, U.S.A.) was examined at concentrations of 2, 5, 10, 25 and 50 μg/m*l* with culture periods of 12, 24, 48, 72 and 96 hr. Culture supernatants were harvested by centrifugation at $800 \times$ g for 10 min, filter-sterilized and stored at -35°C until use.

Bioassay of dog IL-2 activity: Quantitative microassay for dog IL-2 activity was carried out by probit analysis [9]. CTLL-2 maintained in CM containing 10% FCS was used for the bioassay, and recombinant human IL-2 (2,000 U/m*l*, Gillis unit [9]) was used as standard IL-2. IL-2 activity was evaluated as the radioactivity of ^{3}H-thymidine incorporated into CTLL-2. The results were expressed as the mean counts per minute (cpm) for triplicate cultures.

Heat treatment: Culture supernatants obtained under the optimal conditions were heated in a water-bath at 55, 60, 65 and 70°C for 15, 30 and 60 min. IL-2 activity was assayed before and after the treatment.

pH sensitivity: The culture supernatant was precipitated with 85% saturated ammonium sulphate with gentle stirring at 4°C overnight. The precipitate was collected by centrifugation at $800 \times$ g for 20 min, then dissolved in buffered solutions, such as 0.05 M Na_2HPO_3-citric acid (pH 3–6), 0.05 M phosphate buffered solution (PBS, pH 7.2) and 0.05 M Na_2CO_3-NaHCO$_3$ buffered solution (pH 8–12). Samples were then exposed to each buffered solution at 4°C for 5 hr, dialyzed against a 100-fold volume of 0.9% saline and sterilized. IL-2 activity was assayed

before and after the treatment.

Trypsin exposure: The obtained precipitate containing dog IL-2 was dissolved in 0.9% saline and dialyzed against a 100-fold volume of the saline at 4°C for 24 hr, and then treated with trypsin at concentrations of 0.01, 0.05 and 0.1% at 38°C for 15, 30 and 60 min. After the treatments, trypsin activity was inhibited by the addition of FCS made up to 10%. Each sample was sterilized. IL-2 activity was assayed before and after the treatment.

Gel filtration: The molecular weight (MW) of dog IL-2 was determined by a gel filtration method [11] with slight modifications. The precipitate mentioned above was dissolved in the required volume of 0.9% saline, and dialyzed against a 100-fold volume of the saline at 4°C for 24 hr. This sample was applied onto a Sephadex G-100 superfine (Pharmacia, Uppsala, Sweden) column (1.5 × 60 cm) equilibrated with 0.9% saline. The sample was eluted with the same solution at a flow rate of 18 ml/hr at 4°C. The fractionated eluations were collected and diluted 5 times with CM. After the filter-sterilization each activity was assayed, and MW of dog IL-2 was estimated from the number of fractions which indicated high activity. MW markers (Pharmacia, Uppsala, Sweden) used were bovine serum albumin (67K), ovalbumin (43K), chymotrypsinogen-A (25K) and ribonuclease (13.7K).

Long-term culture of lymphocytes: The isolated PBL were adjusted to 2×10^6 cells/ml in CM containing 10% horse serum (HS, prepared from a clinically healthy foal in our laboratory) and PHA-P of 10 μg/ml, then incubated at 38°C in humidified 5% CO_2 for 48 hr. PBL were then washed by centrifugation at 200 × g with PBS 3 times and resuspended to 2 to 5×10^5 cells/ml in PHA-P-free fresh CM containing dog IL-2 at 30 to 100 U/ml. Culture passages were performed based on the conditions of cell proliferation. In every culture passage, cell viability was determined by the trypan blue dye exclusion test, and compositions of lymphocytes and other leukocytes were microscopically examined by Giemsa stain.

Phenotypic analysis of cultured cells: Cytospin preparations of cultured cells were fixed with formalin acetone buffered solutions. Non-specific esterase (α-naphtyl acetate esterase: ANAE) activity was detected according to the method of Knowles *et al.* [17]. Antigens expressed on the cell surface membrane were examined immunohistochemically by indirect or avidin-biotin-complex (ABC) peroxidase techniques [14, 21] to determine the subpopulation of cultured lymphocytes. Sera or mouse monoclonal antibodies (MoAb) used as primary antibodies were as follows: Anti-dog thymocyte rabbit serum (ATS) [21], anti-dog Thy-1 MoAb (αThy-1, Serotec, Oxford, U.K.), anti-dog immunoglobulin rabbit IgG (αIg, Bethyl Lab., Montgomery, U.S.A.), anti-granulocyte/monocyte MoAb (αGM, DH59B, VMRD Inc., Pullman, U.S.A.), and anti-major histocompatibility complex class II MoAb (αMHC II, TH14B, VMRD Inc., Pullman, U.S.A.). As secondary antibodies, anti-rabbit IgG peroxidase-conjugated goat IgG (Bethyl Lab., Montgomery, U.S.A.) was used against ATS and anti-dog Ig

rabbit IgG for indirect technique, and anti-mouse IgG+IgM biotinylated goat IgG (Tago Inc., Burlingame, U.S.A.) was used against MoAb for the ABC technique with a Vectastain kit (Vector Lab., Burlingame, U.S.A.).

Cytostasis test: Both fresh PBL and long-term cultured cells with dog IL-2 (passage at the 10th to 12th) from 3 intact and 3 CTVS-sensitized dogs were submitted to cytostasis test as the effector cells. Removed CTVS tissue was digested with 0.1% trypsin PBS. Floated CTVS cells were washed, suspended in the freezing medium (CM containing 10% HS, 0.5 M sucrose and 1.5 M DMSO) and stocked in liquid nitrogen (−196°C). In the cytostasis test, CTVS cells were thawed rapidly at 37°C, washed and adjusted to 2×10^5 cells/ml with CM containing 10% HS. Fresh PBL and cells long-term cultured with dog IL-2 were adjusted to 4×10^6 cells/ml in the same medium. Triplicate cultures were done in a 96-well culture plate. One hundred μl of CTVS cell suspension was placed in each well, and an equal volume of lymphocyte suspension was added. The culture was maintained at 38°C in 5% humidified CO_2 for 18 hr, and then 0.5 μCi of ^3H-thymidine was added to each well. After reincubation for 6 hr, cells were harvested, and the radioactivities of the incorporated ^3H-thymidine were measured by a liquid scintillation counter. Cytostasis was evaluated as follows:

Cytostasis = [T]cpm/[T+E]cpm

[T]cpm: mean cpm value in single culture of target cells
[T+E]cpm: mean cpm value in co-culture of target cells and effector cells

Statistical analysis: The results were expressed as the mean ± SD. The data obtained were statistically analyzed by Student's *t*-test. P values of less than 0.05 were considered significant.

RESULTS

Optimal conditions for dog IL-2 production: Relationships between IL-2 activities and incubation periods in a PBL population of 2×10^6 cells/ml are shown in Table 1. At all concentrations of PHA-P tested, IL-2 activity tended to increase with time and reached its peak at 48 hr. The maximal value for dog IL-2 activity was observed in the supernatant of canine PBL stimulated with PHA-P at a concentration of 10 μg/ml for 48 hr. Dog IL-2 for subsequent was therefore prepared as a supernatant by incubating a PBL population of 2×10^6 cells/ml cultured with 10 μg/ml of PHA-P at 38°C for 48 hr. However, the amount of IL-2 produced was differed remarkably among experimental dogs (n=6: mean=280 U/ml, maximal=660 U/ml, minimal=55 U/ml).

Heat stability: Dog IL-2 activity was not changed after heating at 55°C and 60°C for 60 min. However, it was inhibited significantly by heating at 65°C and 70°C over 15 min (Fig. 1).

pH stability: Dog IL-2 activity was relatively stable at acidity between pH 5 to 9. It decreased significantly when treated with buffered solutions with a pH below 4 or over 10 (Fig. 2).

Table 1.   Dog interleukin-2 (IL-2) activity in culture supernatants of canine peripheral blood lymphocytes (PBL) stimulated with phytohemagglutinin-P (PHA-P)

PHA-P concentration (μg/ml)	Incubation periods (hr)				
	12	24	48	72	96
2	994±442[a]	1,332±667	2,023±881	1,078±433	1,021±751
5	776±413	974±365	2,241±976	967±454	987±512
10	1,877±786	3,247±813	5,977±953	2,237±1,298	1,697±495
25	1,383±761	1,544±325	3,155±956	2,008±543	1,272±827
50	1,644±670	2,008±997	3,877±1,232	3,202±1450	1,943±758

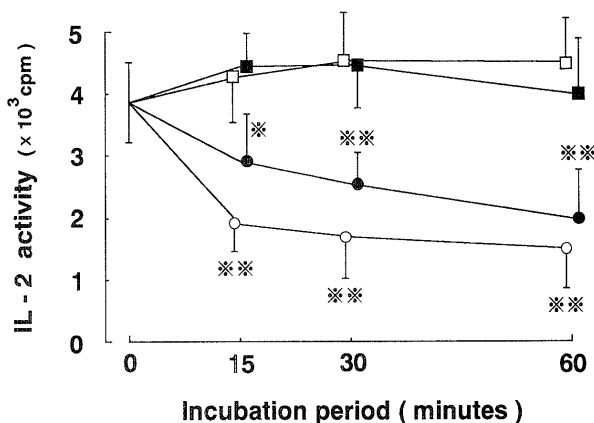a) Values are expressed as mean counts per minute (cpm) of the incorporated ^{3}H-thymidine (n=6, mean ±SD).

Fig. 1.   Heat stability of dog IL-2 activity (-■-: 55°C, -□-: 60°C, -●-: 65°C, -○-: 70°C: n=6, mean ± SD). IL-2 activity was stable at 55°C and 60°C, but not at 65°C or 70°C. Statistical difference were compared with cpm value before heating (**: p<0.01, *: p<0.05). For key see Table 1.
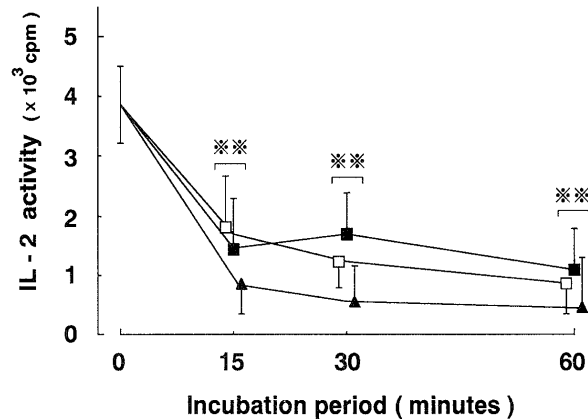

Fig. 3.   Trypsin sensitivity of dog IL-2 activity (-▲-: 0.1%, -□-: 0.05%, -■-: 0.01%, n=6, mean ± SD). IL-2 activity was significantly inhibited by exposure to a concentration of 0.05% trypsin for 15 min. Statistical differences were compared with the cpm value before trypsin exposure (**: p<0.01).
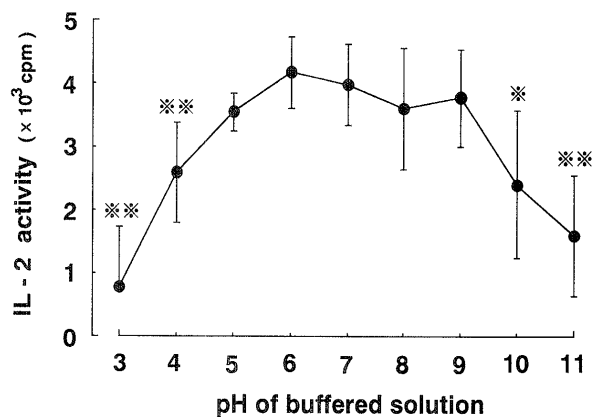

Fig. 2.   pH stability of dog IL-2 activity (n=6, mean ± SD). IL-2 activity was relatively stable from pH 5 to 9, but unstable under pH 4 or over pH 10. Statistical difference were compared with the cpm value at pH 7.2 (**: p<0.01, *: p<0.05).

Trypsin sensitivity: Dog IL-2 activity was almost completely inhibited when treated with 0.01% trypsin for 15 min (Fig. 3).

Molecular weight: Dog IL-2 activity was detected in the fractions with a MW of approximately 28,500 to 33,000, and the peak value was detected in the fraction with a MW of approximately 31,000 (Fig. 4).

Long-term culture of lymphocytes: The mean composition of lymphocytes harvested with Ficoll separation was 77.5% and their viability was more than 90%. Canine PBL started to proliferate favorably in cluster fashion following the addition of dog IL-2 to PBL stimulated with PHA-P. The interval between passages was 2 or 3 days in most cases. When PHA-P-stimulation was not repeated, cell viability began to decrease rapidly at the 4th to 5th passage (< 10%). Unfortunately, the culture failed to continue any longer. In contrast, further favorable proliferation could be maintained by stimulating the cultured cells with PHA-P every 3 passages. Both viability and composition of lymphocytes exceeded 95% at the 5th passage. Most of the cultured cells were small- and medium-sized lymphocytes (Fig. 5). Doubling time for proliferation of cultured cells varied from 18 to 32 hr (mean value: 26.8 hr).

Phenotype of cultured cells: At the beginning of the culture, PBL which contained some contaminating granulocytes reacted to αIg, αGM and αMHCII. At the 5th
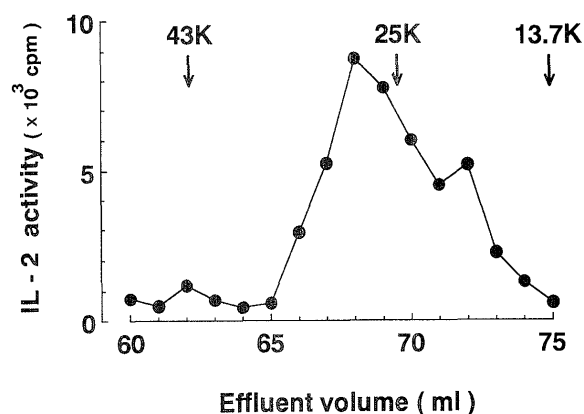
Fig. 4.   Dog IL-2 activity after a Sephadex-G100 gel filtration.
Peak of activity was observed in the fraction with a molecular
weight of approximately 31,000. Arrows show molecular
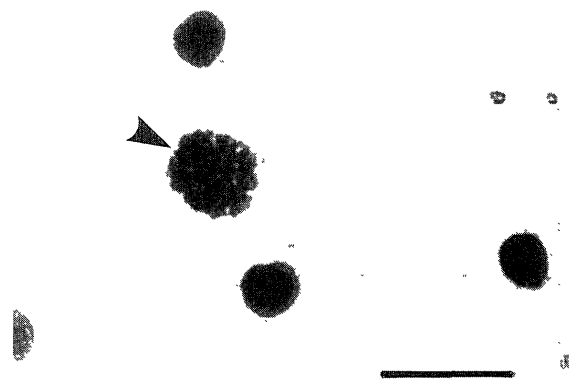weights of marker proteins.

Fig. 5.   Cytopsin preparation of canine long-term cultured
cells with homologous IL-2 stained with Giemsa solution.
Small- and middle-sized lymphocytes were dominant
(Bar=20 μm). Large-sized lymphoblast-like cells were
occasionally observed (arrow head), but no large granular
lymphocytes were detected.

Table 2.   Phenotype of canine cultured cells originating in
peripheral blood at onset, and the 5th and 10th passages

Leukocyte markers	Cultured cells		
	Primary	Passaged	
		5th	10th
Surface antigen			
Thymocyte antigen	+++[a]	++++	++++
Thy-1 antigen	+++	++++	++++
MHC[b] class II antigen	+	±	−
Granulocyte/monocyte antigen	+	±	−
Immunoglobulin	++	±	−
Cytoplasmic enzyme			
α-naphthyl acetate esterase	+++	±	−
Peroxidase	+	−	−

a) The degrees of expression of leukocyte markers were graded
as follows: −; negative, ±; <10%, +; 10–29%, ++; 30–59%,
+++; 60–89%, ++++; ≥ 90%.
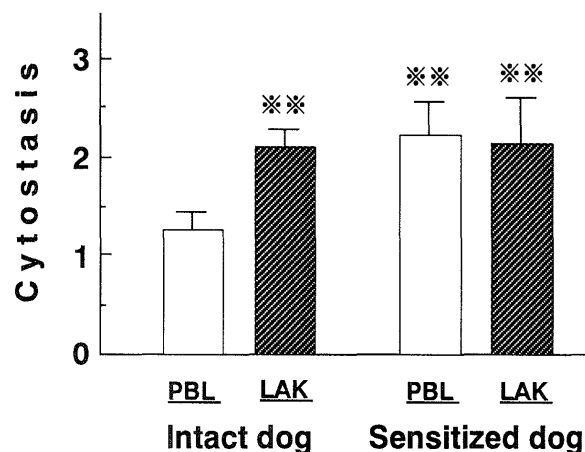b) Major histocompatibility complex.

Fig. 6.   Cytostasis of fresh PBL and long-term cultured cells
from the intact and canine transmissible venereal sarcoma
(CTVS)-sensitized dogs against CTVS cells, respectively
(n=6, mean ± SD). Note the significant difference (**:
p<0.01) between intact PBL and their LAK cells, and
between 2 types of fresh PBL from the intact and CTVS-
sensitized dogs.

and 10th passages, most of the cultured cells (over 90%)
reacted to ATS or αThy-1 and cytoplasmic ANAE activity
was slightly positive and peroxidase activity was negative
(Table 2).

Cytostasis: Cytostasis of fresh PBL and long-term
cultured cells from the intact dogs was 1.23 ± 0.22 and
2.03 ± 0.27 (n=6), respectively (Fig. 6). This indicated
that cytostasis of cultured cells was enhanced significantly
(p<0.01). These lymphocytes were therefore defined as
LAK cells. Cytostasis of fresh PBL and long-term cultured
LAK cells from the CTVS-sensitized dogs was 2.18±0.45
and 2.04±0.54, respectively. Cytostasis of fresh PBL from
the CTVS-sensitized dogs was significantly higher than
that from the intact dogs (p<0.01).

DISCUSSION

Daemen *et al.* [5] reported that growth factor of canine

co-cultured T cells was found in the culture supernatant
from mitogen-stimulated PBL. In our study, IL-2 activity
in culture supernatant of canine PBL stimulated with
PHA-P was examined by means of CTLL-2, which had
been established for IL-2 quantitative assay [9]. In a
preliminary study, canine PBL was cultured with PHA-P
or concanavalin A (ConA) based on a lymphocyte
blastoformation test [21]. IL-2 activity in the supernatant
of canine PBL stimulated with the lectin was examined
under various conditions. The results showed that dog
IL-2 activity was detectable in the supernatant of canine
PBL stimulated with PHA-P and measurable by prolifera-
tion of CTLL-2. In contrast, Cerruti-Sola *et al.* [3]
reported that CTLL-2 showed low response to super-
natant of lectin-stimulated canine PBL. It was therefore

considered that CTLL-2 might respond directly to a trace of PHA-P in the supernatants. However, this apprehension was eliminated by the red blood cell absorption test [2]. ConA-stimulation at various concentrations of 2 to 100 μg/ml induced blastoformation of canine PBL, but no IL-2 activity was detected (Data not shown). The results indicated that ConA-stimulation could not increase IL-2 production to a measurable level in a CTLL-2 proliferation assay.

The highest IL-2 production was observed when a PBL population of 2×10^6 cells/ml was stimulated with PHA-P at a concentration of 10 μg/ml for 48 hr, but longer incubation led to a decrease in IL-2 activity. PHA-P-induced lymphoblasts seemed to consume IL-2 in the supernatant with time. Dog IL-2 activity was inhibited by some treatment such as heating over 65°C, acidification below pH 4, alkalification over pH 10, and trypsin exposure. Dog IL-2 was more susceptible to these treatments than human or rodent IL-2. Dog IL-2 activity was detected in the fraction with a MW of approximately 31,000 by gel filtration. This value is similar to the MW of mouse IL-2 [10]. Dog IL-2 might be detected in a form of aggregation or dimer peptide.

In our laboratory, HS was considered to be suitable for culturing CTVS cells [23] and canine PBL. Intermittent stimulation with PHA-P repeated every 3 passages induced successful long-term culture of canine lymphocytes over 10 passages. It was assumed that PHA-P-stimulation induced IL-2 receptor expressions on the cell surface membrane to promote proliferation in paracrine or autocrine fashions, or that a cluster formation of cultured cells was capable of supporting continued growth. The mean composition of lymphocytes was initially 77.5%, but after the 5th passage, it was more than 95%. Most of the cultured cells were small- and medium-sized lymphocytes. These cells were likely to be T-lineage cells because of their reaction to ATS or αThy-1. αMHCII and αGM used in phenotypic analysis were prepared by immunization of bovine leukocytes. These MoAb were confirmed to react specifically to some peripheral leukocytes or skin Langerhans' cells in canine as well as in other species [6]. The first report concerning long-term culture of canine lymphocytes with IL-2 was published by Hogenesch and Felsburg [13]. They used IL-2 originating in murine or primate to maintain canine lymphocytes from Peyer's patches, established T cell lines.

Cohen reported that CTVS cells were relatively resistant to normal lymphocytes, but susceptible to immune lymphocytes from CTVS-regressed dogs [4]. The results obtained here were similar. Cytostasis in lymphocytes from the intact dogs was greatly increased by long-term culture with dog IL-2. In an additional dye exclusion test, tumor cells incubated with lymphocytes cultured in the presence of dog IL-2 were shown to be dead in the cytostasis test. Therefore, canine lymphocytes long-term cultured with homologous IL-2 could belong to the category of LAK cells described previously [20]. CTVS cells maintained in our laboratory were used to evaluate

the cytostasis of fresh PBL and long-term cultured cells, because both genotypes and phenotypes of these tumor cells were considered to be biologically stable. Examination of cytostasis was carried out on the basis of the inhibition of ^{3}H-thymidine incorporated into CTVS cells. Doubling time for LAK cells was approximately 26 hr, but in a short-term cytostasis test, the radioactivity of ^{3}H-thymidine incorporated into LAK cells was negligible and no significant effect on the evaluation of cytostasis was observed. Therefore, such an evaluation was considered to be possible.

It is known that LAK cells could be divided mainly into ultra-activated NK cells [19] and MHC-unrestricted CTL [25]. Jardine et al. [16] reported that culture of canine PBL with human recombinant IL-2 induced lymphocytes which had the morphology of large granular lymphocytes (LGL) and damaged certain malignant tumors, and that this cytotoxicity reached a significant level on the 4th to 7th day. In contrast, the canine LAK cells referred to here did not resemble LGL microscopically. These differences could be due to culture conditions, for example, the serum added and the incubation period [24, 28].

REFERENCES

1. Alvarez, J. M., Silva, A., and De Landazuri, M. O. 1979. Human T cell growth factor. I. Optimal conditions for its production. J. Immunol. 123: 977–983.
2. Alvarez, J., Rodriguez, J., Lopez-Botet, M., Silva, A., and De Landazuri, M. O. 1981. Removal of PHA from supernatants containing T-cell growth factor. J. Immunol. Methods 40: 289–296.
3. Cerruti-Sola, S., Kristensen, F., Vandevelde, M., and De Weck, A. L. 1984. Interleukin 1- and 2-like activities in the dog. Vet. Immunol. Immunopathol. 6: 261–271.
4. Cohen, D. 1980. In vitro cell-mediated cytotoxicity and antibody-dependent cellular cytotoxicity to the transmissible venereal tumor of the dog. J. Natl. Cancer Inst. 64: 317–321.
5. Daemen, A. J. J. M., Buurman, W. A., Linden, C. J. V. D., Groenewegen, G., and Kootstra, G. 1983/1984. Canine IL-2: Characterization and optimal condition for production. Vet. Immunol. Immunopathol. 5: 247–258.
6. Davis, W. C., Marusic, S., Lewin H. A., Splitter, G. A., Perryman, L. E., McGuire T. C., and Gorham, J. R. 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. Vet. Immunol. Immunopathol. 15: 337–376.
7. Denyer, M. S., Martin, S., and Wardley, R. C. 1987. Cross reactions between porcine, bovine and ovine Interleukin-2 preparations. Res. Vet. Sci. 42: 252–254.

8. Farrar, J. J., Mizel, S. B., Fuller-Farrar, J., Farrar, W. L., and Hilfiker, M. L. 1980. Macrophage-independent activation of helper T cells. I. Production of interleukin-2. *J. Immunol.* 125: 793–798.

9. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A. 1978. T cell growth factor: Parameters of production and a quantitative microassay for activity. *J. Immunol.* 120: 2027–2032.

10. Gillis, S., Mochizuki, D. Y., Conlon, P. J., Hefeneider, S. H., Ramthun, C. A., Gillis, A. E., Frank, M. B., Henney C. S., and Watson, J. D. 1982. Molecular characterization of interleukin 2. *Immunol. Rev.* 63: 167–209.

11. Goitsuka, R., Hirota, Y., Hasegawa, A., and Tomoda, I. 1986. Feline interleukin 2 activity. *Jpn. J. Vet. Sci.* 48: 529–537.

12. Grimm, E. A., Mazumder, A., Zhang, H. Z., and Rosenberg, S. A. 1982. Lymphokine-activated killer cell phenomenon. *J. Exp. Med.* 155: 1823–1841.

13. Hogenesch, H. and Felsburg, P. J. 1989. Development and functional characterization of T cell lines from canine Peyer's patches. *Vet. Immunol. Immunopathol.* 23: 29–39.

14. Hsu, S. M., Raine, L., and Fanger, H. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29: 577–580.

15. Ishikawa, T., Imawari, M., Moriyama, T., Ohnishi, S., Matsuhashi, N., Suzuki, G., and Takaku, F. 1988. Immunotherapy of hepatocellular carcinoma with autologous lymphokine-activated killer cells and/or recombinant interleukin-2. *J. Cancer Res. Clin. Oncol.* 114: 283–290.

16. Jardine, J. H., Jackson, H. J., Lotzava, E., Savary, C. A., and Small, S. M. 1989. Tumoricidal effect of interleukin-2-activated killer cells in canines. *Vet. Immunol. Immunopathol.* 21: 153–160.

17. Knowles, D. M., Hoffman, H. T., Ferrarini, M., and Kunkel, H. G. 1978. The demonstration of acid α-naphthyl acetate esterase activity in human lymphocytes: Usefulness as a T-cell marker. *Cell Immunol.* 35: 112–123.

18. Koike, T., Kudo, T., Otomo, K., and Sakai, T. 1979. Successively transplanted canine transmissible sarcoma. *Gann* 70: 115–118.

19. Lainer, 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.

20. Mittelman, A., Savona, S., Gafney, E., Penichet, K. O.,

Lin, B., Levitt, D., Ahmed, T., Arlin, Z. A., Baskind, P., Needleman, D., Levere, R. D., and Podack, E. R. 1989. Treatment of patients with advanced cancer using multiple long-term cultured lymphokine-activated killer (LAK) cell infusions and recombinant human interleukin-2. *J. Biol. Response Mod.* 8: 468–478.

21. Mizuno, S., Fujinaga, T., Tajima, M., Otomo, K., and Koike, T. 1989. Role of lymphocytes in dogs experimentally rechallenged with canine transmissible sarcoma. *Jpn. J. Vet. Sci.* 51: 86–95.

22. Morgan, D. A., Ruscetti, F. W., and Gallo, R. 1976. Selective *in vitro* growth of T lymphocytes from normal human bone marrows. *Science* 193: 1007–1008.

23. Okamoto, Y., Fujinaga, T., Tajima, M., Hoshi, N., Otomo, K., and Koike, T. 1988. Isolation and cultivation of canine transmissible sarcoma cells. *Jpn. J. Vet. Sci.* 50: 9–13.

24. Ortaldo, J. R., Mason, A., and Overton, R. 1986. Lymphokine-activated killer cells. Analysis of progenitors and effectors. *J. Exp. Med.* 164: 1193–1205.

25. Patel, S. S., Thiele D. L., and Lipsky, P. E. 1987. Major histocompatibility complex-unrestricted cytolytic activity of human T cells. Analysis of precursor frequency and effector phenotype. *J. Immunol.* 139: 3886–3895.

26. Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E., Ettinghausen, S. E., Matory, Y. L., Skibber, J. M., Shiloni, E., Vetto, J. T., Seipp, C. A., Simpton, C., and Reichert, C. M. 1985. Observations on the systemic adminsitration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatis cancer. *New Engl. J. Med.* 313: 1485–1492.

27. Sambhara, S. R. and Belden, E. L. 1988. Bovine interleukin 2: Production and characterization. *Vet. Immunol. Immunopathol.* 18: 165–172.

28. Sawada, H., Abo, T., Sugawara, S., and Kumagai, K. 1988. Prerequisite for the induction of lymphokine-activated killer cells from T lymphocytes. *J. Immunol.* 140: 3668–3673.

29. Svedersky, L. P., Shepard, H. M., Spencer, S. A., Shalaby, M. R., and Palladino, M. A. 1984. Augmentation of human natural cell-mediated cytotoxicity by recombinant human interleukin 2. *J. Immunol.* 133: 714–718.

30. Wagner, H., Hardt, C., Rouse, B. T., Rollinghoff, M., Scheurich, P., and Pfizenmaier, K. 1982. Dissection of the proliferative and differentiative signals controlling murine cytotoxic T lymphocyte responses. *J. Exp. Med.* 155: 1876–1881.