Role of Lymphocytes in Spontaneous Regression of Experimentally Transplanted Canine Transmissible Venereal Sarcoma

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ABSTRACT. Sensitized peripheral blood lymphocytes (PBL) obtained from canine transmissible venereal sarcoma (CTVS)-regressed dogs were more cytotoxic against CTVS cells than non-sensitized PBL from untreated dogs. Cytotoxicity shown by sensitized PBL was inhibited significantly by the addition of anti-major histocompatibility complex (MHC) class II mouse monoclonal antibody as well as that of anti-dog thymocyte rabbit serum. The degree of cytotoxic activity shown by lymphokine activated killer (LAK) cells induced from non-sensitized or sensitized PBL was similar to that of the activity shown by sensitized PBL. These LAK activities were also prohibited by the addition of anti-dog thymocyte rabbit serum. Immunohistochemical examination demonstrated that MHC class II antigens were expressed on the surface membrane of CTVS cells and thymocyte antigens were detected on the surface of the tumor infiltrating lymphocytes. From the results mentioned above, lymphocytes which play a central role in tumor regression are considered to be T cells. These cells might recognize MHC class II antigens on the surface membrane of CTVS cells in tumor regression.—KEY WORDS: canine interleukin-2, lymphokine-activated killer cell, major histocompatibility complex, tumor infiltrating lymphocyte.

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Canine transmissible venereal sarcoma (CTVS) can be transplanted experimentally by allogenic transfer. In such cases, the tumor commonly regresses spontaneously [12, 13]. The reasons for the success of the tumor growth in being engrafted into recipient dogs with dissimilar major histocompatibility complex (MHC) to the donors have not been demonstrated, nor have mechanisms involved in tumor regression been explained in detail [4, 20].

A few *in vitro* analytical studies using mixed lymphocyte-tumor culture [2, 10] and mediations of the MHC molecule have been reported [19]. Several descriptions obtained from morphological or hematological studies indicate that lymphocytes, especially T-like lymphocytes play important roles in the regression of this tumor [1, 11, 15, 18].

In the present study, tumoricidal effects of canine peripheral blood lymphocytes (PBL) and lymphocytes cultured with recombinant human interleukin-2 (IL-2) on CTVS cells were examined in an *in vitro* cytotoxicity test. In addition, surface antigens related to cytotoxicity shown by effector lymphocytes were determined by means of several leukocyte markers. Finally, localization of these antigens was confirmed by immunohistochemistry.

MATERIALS AND METHODS

Animals and transplantation of tumor: Six clinically healthy beagle dogs weighing 7 to 12 kg (3 males and 3 females) were used in this study. Three of the animals had been transplanted with CTVS cells which had been maintained by allogenic serial transfer in our laboratory [13] but the remaining 3 were left untreated. Progressing or stable tumors were used for transplantation. A single cell suspension of CTVS tissue was obtained mechanically and was adjusted to 10^7 to 10^8 cells/m*l*. Three to 5 m*l* of the tumor cell suspension was inoculated subcutaneously into 3 sites each on the right and left hypogastric regions of the dogs. Following spontaneous regression, these animals were called the CTVS-regressed dogs.

Culture medium and isolation of PBL: RPMI-1640 (Gibco Lab., New York, U.S.A.) containing 10 mM of HEPES, penicillin-G (100 U/m*l*) and streptomycin (100 μg/m*l*) was used as a complete medium. The complete medium, supplemented with 10% horse serum (prepared from a clinically healthy foal in our laboratory), was used for the preparation of canine lymphokine activated killer (LAK) cells and in a cytotoxicity test. Canine PBL were isolated as previously described [15]. Fresh PBL from the untreated and CTVS-regressed dogs were called non-sensitized PBL (nsPBL) and sensitized PBL (sPBL), respectively.

Preparation of canine LAK cells: For the preparation of LAK cells, PBL were adjusted to 2×10^6 cells/m*l* in complete medium containing 10% horse serum and 10 μg/m*l* phytohemagglutinin-P (PHA-P, Difco Lab., Detroit, U.S.A.), then incubated at 38°C in humidified 5% CO_2 for 48 hr. The PBL were then washed gently by centrifugation at 200 × g with phosphate buffered saline (pH 7.2) 3 times and resuspended at 2 to 5×10^5 cells/m*l* in PHA-P-free fresh medium containing 100 U/m*l* of recombinant human IL-2, which was produced by the murine fibroblast transferring the human IL-2 gene. LAK cells derived from nsPBL were called nsLAK cells, and those from sPBL, sLAK cells.

Phenotypic analyses of effector and target cells: Cytospin preparations of fresh PBL and LAK cells from the untreated and CTVS-regressed dogs and CTVS cells from the steady stage of tumor growth [1] were fixed in a formalin acetone buffered solution. Surface antigens were detected by immunoperoxidase techniques. Cell markers used for the phenotypic analysis of effector or target cells and cytotoxicity test are listed in Table 1. When mono-

Table 1. Antibodies used in the phenotypic analysis and cytotoxic test

Antibody	Type	Specifity	Souce
ATS[a]	Rabbit polyclonal	Canine thymocytes, lymphocytes in T-cell dependent area of lymph node, spleen, and parts of PBL[f]	prepared in authors' laboratory
αThy-1[b] (F3-20-7)	Mouse monoclonal	Canine T lymphocytes, parts of brain neuron	Serotec (Oxford, UK)
αIg[c]	Rabbit polyclonal	Canine IgG+IgM, B lymphocytes	Bethyl Lab. (Montgomery, U.S.A.)
αMHC II[d] (TH14B)	Mouse monoclonal	MHC class II molecule	VMRD Inc. (Pullman, U.S.A.)
NR[e]	Rabbit whole sera	Negative control	prepared in authors' laboratory

a) Anti-dog thymocyte rabbit serum.
b) Anti-dog Thy-1 mouse monoclonal antibody.
c) Anti-dog immunoglobulin rabbit IgG.
d) Anti-major histocompatibility class II monoclonal antibody.
e) Normal rabbit serum.
f) Peripheral blood lymphocytes.

clonal antibodies were used as the primary antibody, the avidin-biotin peroxidase complex (ABC) technique with a Vectastain ABC kit (Vector Lab., Burlingame, U.S.A.) was undertaken as previously described [8]. When polyclonal antibodies were used as the primary antibody, antigens were detected by the indirect technique [15]. Two hundred cells were counted in every sample and the results were expressed as a percentage of positive cells.

Titration of antibodies used for culture: Antibody titers of various cell markers responsive to effector and target cells were determined by indirect membrane fluorescent antibody techniques [17], because the antibodies were used for viable cells in a cytotoxicity test.

Cytotoxicity test: Both fresh PBL and LAK cells prepared from the untreated and CTVS-regressed dogs were subjected to the cytotoxicity test as the effector cells. CTVS cells were isolated from tissues in the steady stage by trypsinization and were stocked at −196°C until used. For the cytotoxicity test, thawed CTVS cells were resuspended in complete medium containing 10% horse serum to a concentration of 2×10^5 cells/m*l*. Fresh PBL or LAK cells were adjusted to 4×10^6 cells/m*l* in the same medium. Co-culture was performed in triplicate in a 96-well culture plate. One hundred μ*l* of CTVS cell suspension and an equal volume of either PBL or LAK cell suspension were placed in each well. Cell markers were added to each well made up to a quarter of the amount of the antibody titers. Media and normal rabbit serum were added in the same way as the negative control of mouse monoclonal antibody and rabbit polyclonal antibody, respectively. Co-cultures were maintained at 38°C in humidified 5% CO_2 for 18 hr, and then 18.5 kBq of ^{3}H-thymidine was added to each well. After incubation for an additional 6 hr, these cells were collected with a cell harvester, and the radioactivity of incorporated ^{3}H-thymidine was measured in a liquid scintillation counter. A decrease in ^{3}H-thymidine incorporation into CTVS cells means that the effector lymphocytes are cytotoxic.

Histological and immunohistochemical examination: CTVS tissues at various stages were collected and fixed in a 4% periodate-lydine-paraformaldehyde buffered solution. Tissues were trimmed and embedded in paraffin blocks according to routine procedures. Paraffin-sections were stained with hematoxylin and eosin. The number of tumor cells and tumor infiltrating lymphocytes (TIL) in the CTVS tissues was counted in 10 random fields (1.0 mm^2) of 250 μm square areas of each of the tumor specimens using a microscope with a micrometer. The remainding sections were subjected to immunohistochemistry using the ABC [8] or indirect immunoperoxidase technique [15] to identify the population of TIL and the distribution of MHC antigens.

Statistical analysis: The data obtained from the cytotoxicity test were statistically analyzed by Student's *t*-test according to variance equivalence. P value of less than 0.05 was considered significant.

RESULTS

Tumor growth: Tumors were detected as palpable nodules within a week after the inoculation with tumor cells. The tumors occurred in all 18 inoculation sites, developing to hen's egg or first size, and regressed in between 3 and 6 months. Some neoplastic tissues were surgically removed, depending on the stage of tumor growth [1, 11], for histological examination.

Canine LAK cell preparation: After the initial PHA-P-stimulation of PBL from the untreated and CTVS-regressed dogs, both nsPBL and sPBL proliferated favorably. Long-term culture of LAK cells was successful following PHA-P-stimulation every 3 passages. LAK cells at the 10th to 12th passage (approximately 30 days after the culture initiation) were employed as the effector cells in the cytotoxicity test.

Phenotype of effector and target cells and antibody titer: Both fresh nsPBL and sPBL reacted strongly to T

lymphocyte markers such as anti-dog thymocyte rabbit serum (ATS) and anti-dog Thy-1 monoclonal antibody, and their positive ratios were slightly increased to 70%. Following long-term culture, nsLAK cells and sLAK cells reacted remarkably to ATS and anti-Thy-1 mouse monoclonal antibody, and their positive ratios were more than 95%. In contrast, in response to anti-dog immunoglobulin rabbit IgG used as a B lymphocyte marker, an approximate quarter of the nsPBL or sPBL showed positive reactions. In response to anti-MHC class II mouse monoclonal antibody, lymphocytes used as effector cells showed slight reactions (positive ratio: < 10%). Data of positive ratio of effector or target cells and titers of cell markers are summarized in Table 2.

Cytotoxicity: In the cytotoxicity test by means of nsLAK cells, sPBL and sLAK cells, the ^{3}H-thymidine incorporation into CTVS cells was inhibited significantly in comparison to that in the single culture of CTVS cells ($P<0.01$) and cytotoxicities of these lymphocytes were detected. In the test of a mixed culture with tumor cells and nsPBL, propagation of the tumor cells was almost unchanged despite the addition of medium and markers. Cytotoxicities shown by nsLAK cells, sPBL and sLAK cells were significantly inhibited by the addition of ATS, but not by that of anti-Thy-1 mouse monoclonal antibody. In the test with sPBL as effector cells, the addition of anti-MHC class II mouse monoclonal antibody resulted in a significant increase in ^{3}H-thymidine incorporation into CTVS cells. On the other hand, the addition of this monoclonal antibody to nsLAK and sLAK cells had no effect on ^{3}H-thymidine incorporation, and nsLAK and sLAK activities were almost constant. The data are shown in Fig. 1 and Fig. 2.

Histological and Immunohistochemical findings: In growing or steady-state tumors, the tissues were mainly composed of closely packed, round CTVS cells with distinct nucleoli. MHC class II antigens were detected on the surface membrane of tumor cells (Fig. 3A). Some lymphocytes were seen scattered among the round tumor cells. In regressing tumor, many TIL were observed among the tumor cells and acellular connective tissues accompanied by large amounts of collagen were also detected. Most of these TIL strongly reacted to T cell markers (Fig. 3B). CTVS cells surrounded by TIL

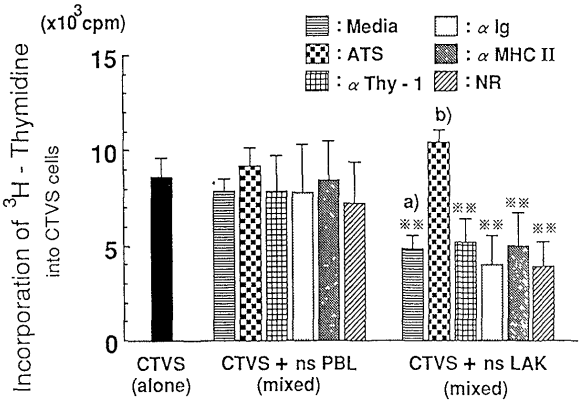

Fig. 1. Cytotoxicity test with nsPBL or nsLAK cells as effector cells against CTVS cells (n=6, mean ± SD). In the test with nsPBL, no cytotoxic activity was detected. When nsLAK cells were used as effector cells, ^{3}H-thymidine incorporation into CTVS cells was significantly decreased in compared to that in the single culture of CTVS cells (a, **: $P<0.01$), and cytotoxic activity was seen. This activity was inhibited by the addition of ATS (b), but not affected by the addition of other cell markers. For key, see Table 1 and Table 2.

Table 2. Positive ratio (%) of several cell markers to effector and target cells and antibody titer

Effector/	Cell markers				
Tumor cells	ATS	αThy-1	αIg	αMHC II	NR
nsPBL[a)]	74.3±9.4[e)]	72.3±9.5	24.9±8.7	8.1±3.5	—
	(12,800)[f)]	(3,200)	(1,600)	(400)	(—)
nsLAK[b)]	96.5±3.3	98.3±1.2	<5	<5	—
	(12,800)	(6,400)	(10)	(10)	(—)
sPBL[c)]	72.6±8.8	67.2±11.5	28.9±12.5	6.5±2.1	—
	(12,800)	(3,200)	(1,600)	(800)	(—)
sLAK[d)]	96.8±2.5	98.8±1.1	<5	<5	—
	(12,800)	(3,200)	(10)	(10)	(—)
CTVS cells	—	—	—	88.3±10.5	—
	(—)	(—)	(—)	(400)	(—)

a) PBL obtained from untreated dogs.
b) Lymphokine-activated killer (LAK) cells induced from nsPBL with recombinant human interleukin-2 (IL-2) at the 10th to 12th passage.
c) PBL obtained from the dogs sensitized by canine transmissible venereal sarcoma (CTVS) challenge.
d) LAK cells induced from sPBL with recombinant human IL-2 at the 10th to 12th passage.
e) Mean±standard deviation, n=6.
f) Antibody titer expressed as reciprocal of highest dilution.
For key see Table 1.

indicated degenerative or necrotic features. Degrees of TIL and tumor cells at each stage of tumor growth are described in Table 3.

DISCUSSION

The *in vitro* cytotoxicity test was performed to examine cell surface antigens of effector cells and target cells involved in tumor regression. Cytotoxicities shown by nsLAK cells, sPBL and sLAK cells were significantly inhibited by the addition of ATS. Effector lymphocytes which played a major role *in vitro* are therefore considered T lymphocytes. No significant change in cytotoxicity was observed in the addition of anti-dog Thy-1 mouse monoclonal antibody. The reason could be explained that ATS is a polyclonal antibody, and this serum can mask various antigens including the T cell receptors expressed on the surface membrane of T cells. Masking of the T cell receptor may prohibit the cytotoxicity as reported in human lymphocytes. [5]. Anti-dog Thy-1 mouse monoclonal antibody reacts to the canine Thy-1 molecule [14]. This molecule might be unrelated to *in vitro* intracellular contact or lymphocyte recognition.

Cohen *et al.* [3, 4] reported that β_2-microglobulin, which was a component of the heavy chain of the canine MHC I molecule [7], was not expressed on the tumor cells. Therefore, no investigation for MHC class I antigen was carried out. In contrast, Yang *et al.* [19] confirmed the expression of MHC class I antigen on the surface membrane of CTVS cells as well as that of MHC class II antigen at each stage of tumor growth. The monoclonal antibody to MHC class II antigen used here was prepared by immunization of bovine leukocytes but the cross

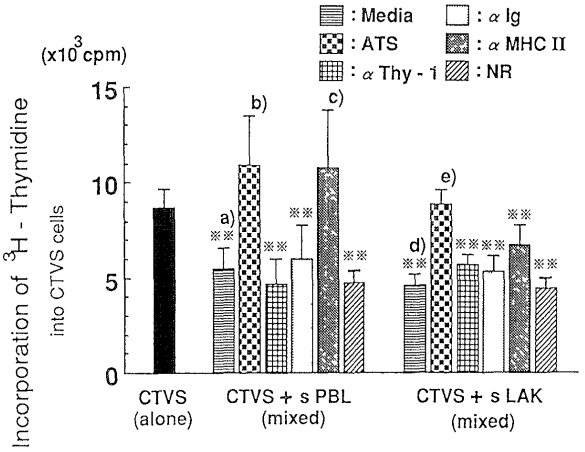

Fig. 2. Cytotoxicity test with sPBL or sLAK cells as effector cells against CTVS cells (n=6, mean ± SD). sPBL indicated cytotoxic activity (a, **: P<0.01) however its activity was significantly inhibited by the addition of ATS (b) and α MHCII (c). Cytotoxicity was observed in sLAK cells (d). sLAK activity was remarkably inhibited by the addition of ATS (e). Caution: Cytotoxicity shown by sPBL was remarkably inhibited by the addition of α MHCII, but, on the other hand, sLAK activity was not affected. For key, see Table 1 and Table 2.

Table 3. Degrees of tumor infiltrating lymphocytes (TIL) and canine transmissible venereal sarcoma (CTVS) cells observed in central and marginal regions of the tumor at each stage of CTVS growth

	Progression		Steady		Regression	
region	TIL	CTVS cells	TIL	CTVS cells	TIL	CTVS cells
center	++	++++	±~+	++++	+++	±~+
margin	+	++++	+	+++	++++	+

Degrees were regarded as follows: ±; <10%, +; 10~29%, ++; 30~59%, +++; 60~89%, ++++; ≧90%.

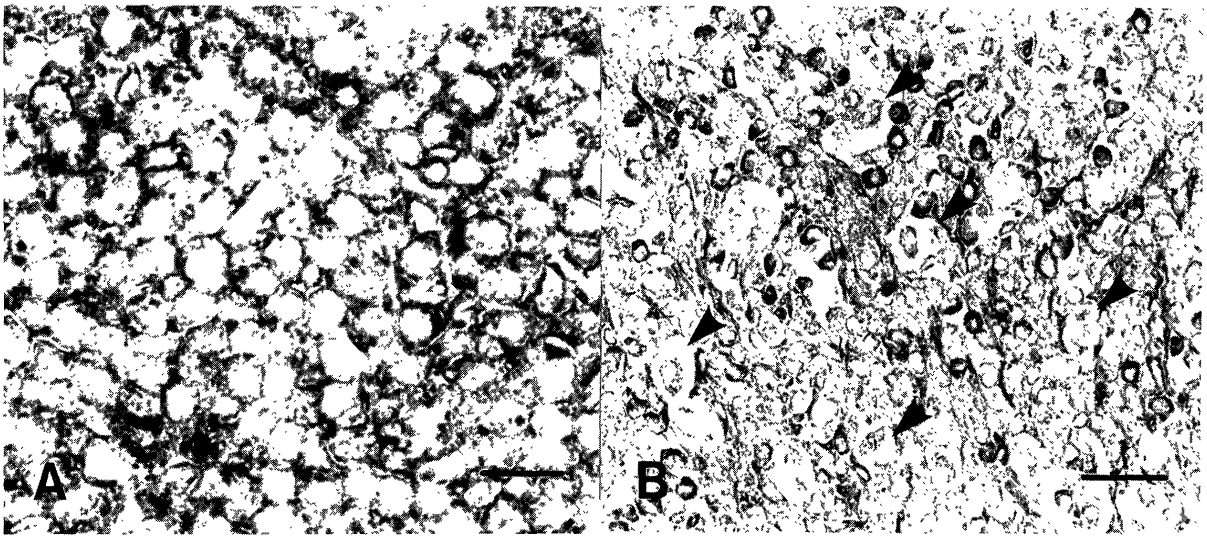

Fig. 3. Immunohistochemical findings for CTVS tissues. A: Most of the CTVS cells strongly reacted with anti-major histocompatibility complex class II mouse monoclonal antibody. B: Tumor infiltrating lymphocytes penetrating regressing tumor tissue. These lymphocytes exhibit thymocyte antigens and were considered to be T cells (Bar = 20 μm). CTVS cells were surrounded by lymphocytes, and tumor cells were degenerative or cytolytic (arrow head).

reactivities have been confirmed in canine and other species [6, 8].

Fresh sPBL exhibited remarkable cytotoxicity in relation to CTVS cells but nsPBL were not cytotoxic. sPBL seemed to have acquired immunity to CTVS cells, reflecting the resistance to a second challenge of the tumor [15]. Circulating sPBL could be precursors of TIL observed in a secondary transplanted tumor. Cytotoxicity shown by sPBL was inhibited by the addition of anti-MHC class II mouse monoclonal antibody as well as by that of ATS. The cytotoxicity shown by sPBL is therefore thought to be dependent on the MHC class II molecule. Immunohistochemistry demonstrated that MHC class II antigens were expressed on the side of CTVS cells and thymocyte antigens on the side of TIL. Similar data are shown by the immunocytochemistry of effector or target cells. In an *in vitro* cytotoxicity test with cell markers, it is considered that ATS reacted with effector cells, and anti-MHC class II antigen mouse monoclonal antibody with target cells.

Chandler and Yang [1] reported that moderate occupancy of B lymphocytes (progressor: 37.3%, steady state: 30.9%, regressor: 26.1%) was observed in the TIL subpopulation. In a case of spontaneous regression of experimentally induced tumor, Hamada *et al.* described how equine T lymphocytes infiltrated into the regressing equine papilloma and that MHC class II antigen positive Langerhans' cells were observed [8].

Generally, in the rejection of a transplanted allograft, helper T cells are activated by recognition of the MHC class II molecule. The helper cells secrete endogenous IL-2 and amplify cytotoxic T cells in a paracrine fashion. We postulate that IL-2-activated lymphocytes could be induced from TIL populations in the same way. Therefore, LAK cells were prepared and their effects on CTVS cells were examined. The results showed that anti-tumor effects on CTVS cells were detected in both nsLAK and sLAK cells. In the dogs bearing CTVS, cytotoxic T cells might be induced through IL-2 and IL-2 receptor pathways as reported in dogs [9]. Cytotoxicities shown by nsLAK and sLAK cells were not inhibited by the addition of anti-MHC class II mouse monoclonal antibody. LAK cells referred here are considered to be MHC-unrestricted cytotoxic T cells [16]. Further study will be necessary in order to identify subsets of canine T lymphocytes.

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REFERENCES

1. Chandler, J. P. and Yang, T. J. 1981. Canine transmissible venereal sarcoma: Distribution of T and B lymphocytes in blood, draining lymph nodes and tumors at different stages of growth. *Br. J. Cancer* 44: 514–521.
2. 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.
3. Cohen, D., Shalv, A., and Krup, M. 1984. Lack of β_2-microglobulin on the surface of canine transmissible venereal tumor cells. *J. Natl. Cancer Inst.* 72: 395–401.
4. Cohen, D. 1985. The canine transmissible venereal tumor: A unique result on tumor progression. *Adv. Cancer Res.* 43: 75–112.
5. David, V., Bourge, J. F., Guglielmi, P., Mathieu-Mahul, D., Degos, L., and Bensussan, A. 1987. Human T cell clones use a CD3-associated surface antigen recognition structure to exhibit both NK-like and allogeneic cytotoxic reactivity. *J. Immunol.* 138: 2831–2836.
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. Feltz, M. J. M., Korput, H. A. G. M., Giphart, M. J., and Westbroek, D. L. 1984. Dog MHC class I antigens contain β_2-microglobulin. *Exp. Clin. Immunogenet.* 1: 56–59.
8. Hamada, M., Takechi, M., and Itakura, C. 1992. Langerhans' cells in equine papillomas and normal skin. *Vet. Pathol.* 29: 152–160.
9. Helfand, S. C., Modiano, J. F., and Nowell, P. C. 1992. Immunophysiological studies of interleukin-2 and canine lymphocytes. *Vet. Immunol. Immunopathol.* 33: 1–16.
10. Hess, A., Cunningham, B., Bennett, T., and Epstein, R. 1975. *In vitro* correlates of the *in vivo* course of the canine transmissible venereal tumor studied by mixed lymphocyte-tumor cultures. *Transplantation Proc.* 7: 507–511.
11. Hill, D. L., Yang, T. J., and Wachtel, A. 1984. Canine transmissible venereal sarcoma: Tumor cell and infiltrating leukocyte ultrastructure at different growth stages. *Vet. Pathol.* 21: 39–45.
12. Karlson, A. G. and Mann, F. C. 1952. The transmissible venereal tumor of dogs: Observations on forty generations of experimental transfers. *Ann. New York Acad. Sci.* 53: 1197–1213.
13. Koike, T., Kudo, T., Otomo, K., and Sakai, T. 1979. Successively transplanted canine transmissible sarcoma. *Gann* 70: 115–118.
14. McKenzie, J. H. and Fabre, J. W. 1981. Studies with a monoclonal antibody on the distribution of Thy-1 in the lymphoid and extracellular connective tissues of the dogs. *Transplantation* 31: 275–282.
15. 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.
16. Patel, S. S., Thiele, D. L., and Lipsky, P. E. 1987. Major histocompatibility complex-unrestricted cytolytic activity of human T cells. *J. Immunol.* 139: 3886–3895.
17. Tajima, M., Fujinaga, T., Okamoto, Y., Otomo, K., and Koike, T. 1990. Relationships between mitogen receptors in peripheral blood lymphocytes and blastogenic responses to mitogen. *Res. Vet. Sci.* 48: 1–5.
18. Trail, P. A. and Yang, T. J. 1982. Canine transmissible venereal sarcoma: Quantitation of T-lymphocyte subpopulations during progressive growth and spontaneous tumor regression. *J. Natl. Cancer Inst.* 74: 461–467.

19. Yang, T. J., Chandler, J. P., and Dunne-Anway, S. 1987. Growth stage dependent expression of MHC antigens on the canine transmissible venereal sarcoma. *Br. J. Cancer* 55: 131–134.

20. Yang, T. J. 1988. Immunobiology of a spontaneously regressive tumor, the canine transmissible venereal sarcoma (review). *Anticancer Res.* 8: 93–96.