

SHORT COMMUNICATION

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Eberhard Burkhardt**Natural killer (NK) and lymphokine-activated killer (LAK) cell functions from healthy dogs and 29 dogs with a variety of spontaneous neoplasms**Received: 2 April 2004 / Accepted: 17 May 2004 / Published online: 2 September 2004
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Abstract To investigate natural killer (NK) and lymphokine-activated killer (LAK) cell functions from 10 healthy dogs and 29 dogs with a variety of spontaneous neoplasms, large granular lymphocytes (LGLs) from blood samples were separated by a 58.5% Percoll density gradient. LGLs were stimulated with a low dose of recombinant human interleukin 2 (rhIL-2) for 7 days. Cytotoxicity of effector cells against the susceptible CTAC cell line was measured before and after stimulation. Compared with those before stimulation, the percentage of LGLs after stimulation with rhIL-2 was found to be significantly increased ($P < 0.01$) in both dogs with tumors and controls. However, the increase was significantly higher in control animals, indicating a defect in proliferation ability of NK cells in canine tumor patients. After stimulation with rhIL-2, lymphokine-activated killer (LAK) cell activity in dogs with tumors was significantly lower ($P < 0.01$) when compared with controls. Reduced cytotoxicity of rhIL-2-activated NK cells in dogs with tumors seems to be attributable to the presence of a diminished proliferative capacity of NK cells and a decreased ability of LAK cells to lyse target cells. Further knowledge of the precise function of IL-2-activated NK cells in dogs with tumors may help to optimize new and therapeutically beneficial treatment strategies in canine and human cancer

patients. Our findings suggest that the dog could also serve as a relevant large animal model for cancer immunotherapy with IL-2.

Keywords Dog · Interleukin 2 · Lymphokine-activated killer cell activity · Natural killer cells · Tumor disease

Introduction

Interleukin 2 (IL-2) plays a central role in the regulation of cell-mediated immunity through stimulation and proliferation of T cells, natural killer (NK) cells, and activated B cells. Human and murine lymphocytes can be activated by IL-2 to become cytotoxic, with a wide-ranging lymphokine-activated killer (LAK) cell activity, able to lyse both freshly NK cell-resistant isolated autologous and cultured allogenic tumor cells [8, 9]. It has been shown that most LAK cell activity mediated by blood lymphocytes is attributable to stimulation of NK cells by IL-2 [11]. Both NK and LAK cell activity appear to have an active role in resistance to cancer development and spread.

The systemic administration of LAK cells and/or recombinant human IL-2 (rhIL-2) has shown biologic and therapeutic efficacy in the treatment of human patients with advanced neoplasms [22]. However, further studies are still necessary to determine the optimal dosages and combinations of chemotherapeutic agents, timing of surgery, the use of immune biologic response modifiers such as IL-2, and to understand the mechanisms used by some tumors to escape from immune attack [3, 17, 23].

Due to a high prevalence of spontaneous neoplasms, dogs are the most frequent tumor patients in veterinary practice, but little is known about the cellular immune response against neoplastic cells in this species. It has been shown recently that NK cell activity is depressed in dogs with certain types of tumors [6, 20]. IL-2 is able to activate in vitro cytotoxic function in peripheral blood

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lymphocytes from healthy dogs [10, 12, 15, 21]. However, information about LAK cell activity in canine tumor patients is limited. After stimulation with rhIL-2, LAK cell activity was not found to be significantly increased in five dogs with lung tumors when compared to values before stimulation [15]. LAK cells from dogs implanted with transmissible venereal sarcoma showed serial growth in rhIL-2-containing culture medium for at least 2 weeks without losing in vitro antitumor activity [16].

In the dog, histologic behavior of tumors, patterns of tumor progression, metastasis, and clinical signs of these spontaneous neoplasms are often identical to those in human patients. Because of this, numerous therapeutic approaches used for treatment of human cancer have been adapted to canine patients. The use of rhIL-2 and/or LAK cells alone or in combination with other therapies might be an additional helpful tool in the treatment of canine neoplasms. The dog may also serve as an additional large animal model to improve cancer (immuno-) therapy in man.

Most of the publications to date are based on NK and LAK cell activity measurements performed with the ⁵¹Cr-release assay (CRA). In the present study, the colorimetric rose bengal assay (RBA) was used to measure cytotoxicity [7]. It has been previously shown that the RBA measures both apoptotic and necrotic cells and therefore provides a more sensitive tool to assess cytotoxicity in dogs [24].

The purpose of this study was to assess the influence of tumor disease on IL-2-activated NK cells and to better support the future use of low-dose rhIL-2 as an immunotherapeutic agent in dogs. To our knowledge, this is the first study in which LAK cell activity was examined in dogs with a variety of different tumors.

Material and methods

Animals

A total of 29 dogs with different benign and malignant spontaneous neoplasms at different stages were examined (3 benign and 26 malignant neoplasms; Table 1). The average age was 7 years. Only previously untreated dogs were considered for this study. The control group

included ten healthy adult beagle dogs, age-matched with the patient population and vaccinated against leptospirosis, distemper, parvovirus, rabies, and infectious canine hepatitis. Blood sampling was not done before 3 months after vaccination. Diagnoses of tumors were confirmed by histologic examination according to the WHO International Histological Classification of Tumors of Domestic Animals.

Preparation of large granular lymphocytes (LGL)

Lymphocytes were isolated from heparinized blood samples by a 58.5% Percoll (Amersham Biosciences, Sweden) density gradient (v/v, specific gravity 1.073), as previously described [7]. Heparinized venous blood was mixed with two parts RPMI 1640 (PAA, Austria) culture medium containing 10,000 U/ml penicillin and 10,000 µl/ml streptomycin (Biochrom KG, Germany) and layered over Percoll. Following centrifugation at 400 g for 25 min, whole blood mononuclear cells were removed from the gradient interface and washed in RPMI 1640. Separated cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum and cultured in plastic culture dishes at 37°C in a humidified 5% CO₂ atmosphere for 1 h to remove adherent cells.

Stimulation of LGL with rhIL-2

Nonadherent effector cells at a concentration of 5.0×10⁶ cells/ml were stimulated twice (day 0 and 3) with 100 IU/ml rhIL-2 and cultured for 7 days. At day 3, old culture medium was removed and fresh culture medium with 100 IU/ml rhIL-2 was added. Cultures were placed into 25-cm² tissue culture flasks and incubated at 37°C in a humidified 5% CO₂ atmosphere.

Morphologic evaluation of LGL

Freshly isolated and rhIL-2-activated lymphocytes were stained with trypan blue to assess viability, counted in a hemocytometer, and diluted to appropriate concentrations for further assays. Additionally, 2.0×10⁵ cells were centrifuged onto microscope slides using a cytospin

Table 1 Different tumor groups

Tumor type	n	Median age (year)	Mean age (year)	Minimum (year)	Maximum (year)
Mammary adenomas	2	10.0	10.0	10.0	10.0
Mammary carcinomas	2	7.0	7.0	6.0	8.0
Other carcinomas	3	8.0	7.5	2.0	12.0
Malignant mixed mammary tumors	3	9.0	9.5	8.0	11.0
Lymphomas	7	6.5	6.5	5.0	8.0
Myeloid neoplasms	4	4.5	4.0	1.0	6.0
Other sarcomas	5	12.0	10.0	5.0	13.0
Other tumors ^a	3	5.0	4.5	2.0	7.0

^aIncluding one benign tumor

centrifuge (Shandon Elliot Cytospin, Germany). Cells were stained with May-Gruenwald-Giemsa according to a standard manual procedure. Both freshly isolated and rhIL-2-activated NK cells were identified morphologically as LGLs with a large, eccentric, often indented nucleus and azurophilic granules in the cytoplasm [4].

Cytotoxicity of freshly isolated NK cells and rhIL-2-activated NK cells

The canine thyroid adenocarcinoma cell line (CTAC) was used as the target cell line [13]. Vero cells were used as the negative control. Cytotoxicity of effector cells was measured before and after the stimulation with rhIL-2. For determination of NK cell activity (natural cytotoxicity), only nonadherent blood mononuclear cells were used as effector cells.

For the measurement of cytotoxicity, the colorimetric RBA was used [7]. Both freshly isolated NK cells and rhIL-2-activated NK cells were coincubated with CTAC and Vero cells in rigid 96-well flat-bottomed plates at effector-to-target cell ratios of 50:1 and 25:1. CTAC or Vero cells without effector cells served as controls. Triplicate cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. After 14 h incubation, lysed target cells and effector cells were removed. The remaining adherent target cells were exposed to the rose bengal dye for 3 min. After three washes, rose bengal dye was released into the supernatant with ethanol, and the optical density (OD) was measured using an ELISA reader.

Cytotoxicity was calculated from the mean of each set of triplicates using the formula:

$$\text{Cytotoxicity in \%} = 100 - \left(\frac{\text{OD test}}{\text{OD control}} \times 100 \right)$$

Statistical analysis

Data from the separation of LGLs and cytotoxicity against CTAC cells were analyzed by the two-factorial variance analysis with repeated measures. Medians, and first and third quartiles were calculated and are shown in the figures. SPSS version 10 for Windows was used for statistical analysis. Values with $P < 0.01$ were considered to be statistically significant.

Results

Preparation of LGLs

After stimulation with rhIL-2, the percentage of LGLs in cell preparations was significantly increased ($P < 0.01$) in both dogs with tumors and healthy controls. However, the increase of LGLs in dogs with tumors was

significantly lower ($P < 0.01$) than in healthy controls (Fig. 1).

Before stimulation with rhIL-2, the median percentage of LGLs in cell preparations from dogs with tumors was 2.5% (mean 2.7%; range 1.0–5.7%), which was comparable to healthy controls (median 2.9%; mean 2.8%; range 1.0–4.0%). After stimulation with rhIL-2, the median percentage of LGLs was only 5.5% (mean 6.7%; range 1.0–16.5%) in dogs with tumors, while it was 11.0% in healthy controls (mean 17.6%; range 6.5–48.5%). Cells stimulated with rhIL-2 were mainly composed of small- to middle-sized lymphocytes and typical LGLs (Fig. 2).

Cytotoxicity of freshly isolated NK cells and rhIL-2-activated NK cells

In Figs. 3 and 4, cytotoxicity of freshly isolated NK cells and rhIL-2-activated NK cells at effector-to-target cell ratios of 50:1 and 25:1 in dogs with tumors and healthy controls are shown.

After stimulation with rhIL-2, the overall cytotoxicity at both effector-to-target cell ratios was found to be significantly increased ($P < 0.01$) in dogs with tumors and healthy controls. However, the increase in cytotoxicity in dogs with tumors was significantly lower ($P < 0.01$) than in controls.

In dogs with tumors, median cytotoxicity before stimulation at an effector-to-target cell ratio of 50:1 was 12.1% (mean 15.1%; range 1.9–31.0%), while it was

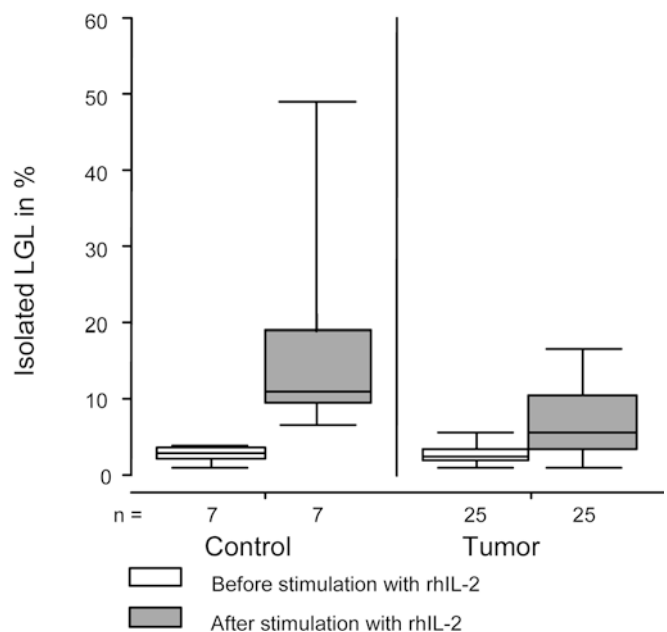


Fig. 1 Percentage of isolated LGLs before and after stimulation with rhIL-2; two-factorial variance analysis with repeated measures; median, and first ($Q1$) and third ($Q3$) quartile, minimum and maximum; mean effect group (tumor vs healthy control), mean effect time (before vs after stimulation) and interaction group \times - time: $P < 0.01$

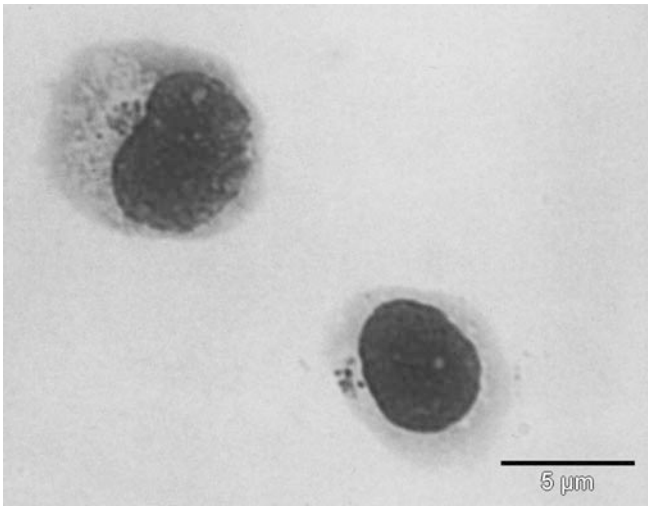


Fig. 2 Lymphokine-activated killer (LAK) cells with the typical morphology of LGLs

only 16.6% after stimulation (mean 27.7%; range 3.0–93.6%). In contrast, median cytotoxicity in healthy controls dramatically increased from 9.8% (mean 8.6%; range 1.5–13.4%) before stimulation, to 73.8% (mean 74.9%; range 55.7–89.9%) after stimulation (Fig. 3).

At an effector-to-target cell ratio of 25:1, cytotoxicity in dogs with tumors after stimulation (median 13.1%; mean 26.5%; range 2.6–92.5%) was comparable to cytotoxicity before stimulation (median 17.1%; mean 17.3%; range 0.3–75.5%). However, median cytotoxicity in healthy controls increased from 11.8% (mean 11.8%; range 3.4–22.2%) before stimulation, to 54.8% (mean 56.5%; range 20.8–94.2%) after stimulation (Fig. 4).

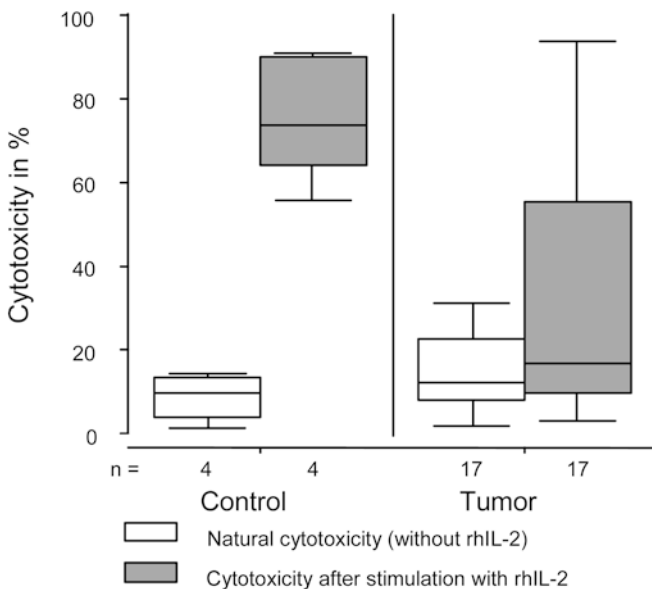


Fig. 3 Cytotoxicity before and after stimulation with rhIL-2 at an effector-to-target cell ratio of 50:1; two-factorial variance analysis with repeated measures; median, Q1, Q3, minimum and maximum; mean effect group (tumor vs healthy control), mean effect time (before vs after stimulation), and interaction group \times time: $P < 0.01$

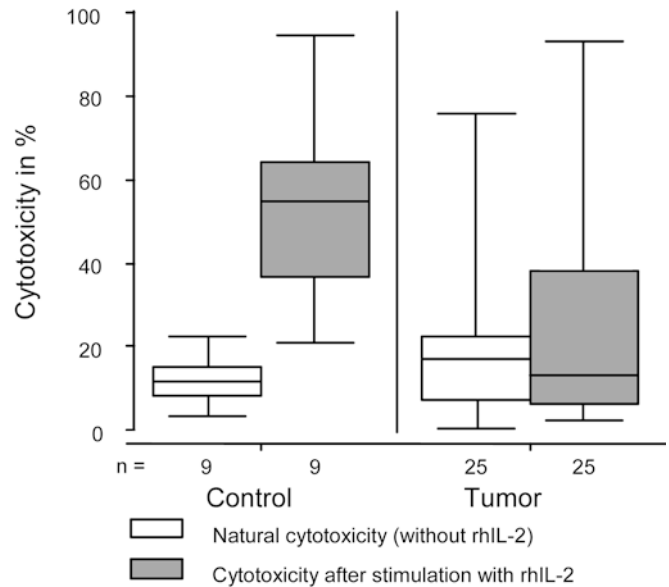


Fig. 4 Cytotoxicity before and after stimulation with rhIL-2 at an effector-to-target cell ratio of 25:1; two-factorial variance analysis with repeated measures; median, Q1, Q3, minimum and maximum; mean effect group (tumor vs healthy control), mean effect time (before vs after stimulation), and interaction group \times time: $P < 0.01$

No cytotoxicity against Vero cells (negative control) in either dogs with tumors or healthy controls was observed before and after the stimulation with rhIL-2 (data not shown).

Discussion

Natural killer and LAK cells, through the use of immune biologic modifiers, have been demonstrated to have a therapeutic role in the treatment of human cancers. Complete regression was seen in human patients after adoptive immunotherapy [22].

So far, only little is known about LAK cell activity in dogs with tumors. Previous studies have shown impaired cellular immune functions such as NK cell activity or mitogen-induced lymphocyte proliferation in dogs with different tumors [5, 6, 20, 25]. These findings supported the hypothesis that cellular immune defects are also present in canine tumor patients.

In the present study, rhIL-2 was able to increase the number of LGLs from both dogs with tumors and controls, after stimulation for 7 days. This demonstrates that rhIL-2 is also able to stimulate proliferation of NK cells in dogs with tumors. However, the increase of LGLs after stimulation with rhIL-2 was significantly lower in dogs with tumors. The results of this study suggest that reduced cytotoxicity of rhIL-2-activated NK cells in dogs with tumors is partly attributable to the presence of a diminished proliferative capacity of NK cells. In addition, the cytotoxic response of rhIL-2-activated NK cells from canine tumor patients seems to be depressed since the increase of LGLs after

stimulation with rhIL-2 did not lead to increased cytotoxicity of these cells.

These observations support the hypothesis that there is also a defect in LAK cell activity in dogs with tumors, as previously described in dogs with lung tumors [15]. Reduced LAK cell activity was also observed in human patients with bronchial carcinoma, advanced gastrointestinal cancer, breast carcinoma, and melanoma [1, 2, 14, 18]. In patients with melanoma, the decrease in LAK cell activity was also correlated with increasing stage of the tumor disease [1].

Complete tumor regression in dogs with melanoma was observed after treatment with tumor necrosis factor (TNF) and IL-2 [19], whereas in other tumor types such as squamous cell or mammary carcinoma, there was no regression at all. Modified response to TNF and IL-2 in combination with an impaired activity of NK cells in dogs with certain tumor types may explain the failure of immunotherapy in some canine tumor patients. In the present study, there was a considerable variance of values in dogs with tumors, which also supports the hypothesis that there might be differences between different histologic types of tumors. In a previous study, decreased NK cell activity was seen in dogs with advanced tumor disease [6]. It remains unknown whether there is also a modified response to IL-2 with increasing tumor load. This needs to be verified in a follow-up study. Further studies are also needed to clarify whether there is also a serum suppressor factor or an elevated prostaglandin E2 production by monocytes present in dogs with tumors, as has been described in human patients [1, 2].

As NK cell–restricted specific markers have not yet been described for dogs, LGLs were identified according to their morphology when stained manually with May-Gruenwald-Giemsa. Cytotoxicity against Vero cells did not increase after stimulation in both dogs with tumors and controls. These target cells appear to be very resistant to the activity of rhIL-2–activated NK cells from the dogs. Although the CTAC cell line is considered to be susceptible for both canine NK and LAK cells [4, 6, 7, 10, 12, 24], the authors cannot show that the results from this cell line are representative for other established cell lines.

In this study, the treatment of canine NK cells with a low dose of rhIL-2 significantly increased the percentage of LGLs in cell preparations and the cytotoxicity of these cells in both dogs with tumors and controls. Therefore, the dog could serve as a relevant large animal model for cancer immunotherapy with IL-2. Further knowledge of the precise function of IL-2–activated NK cells in dogs with tumors may help to optimize new and therapeutically beneficial treatment strategies in canine and human cancer patients.

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