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Clinical and immunological effects of human recombinant interleukin-2 given by repetitive weekly infusion to normal dogs

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Abstract. Four normal adult dogs received two consecutive weekly cycles of human recombinant interleukin-2 (IL-2) by continuous infusion for 4 days/week. The dose of IL-2 given to each dog was 3×10^6 units m^{-2} day^{-1}. Toxicities consisted of mild vomiting, diarrhea, and lethargy to varying degrees in all the dogs. These side-effects were reversed when the treatment was discontinued. Fever, tachypnea, and weight gain were not seen. A marked lymphocytosis and eosinophilia developed in all dogs after completion of each course of IL-2 (resulting in a more than sevenfold increase in each cell type) and persisted for more than 1 month in some. Fresh peripheral blood lymphocytes (PBL) obtained during this lymphocytosis mediated enhanced in vitro lysis of a natural-killer-cell-sensitive canine tumor cell line (CTAC). The in vitro proliferative responses of these same PBL to IL-2 could be detected earlier, progressed faster, and involved more cells than PBL tested prior to IL-2 infusion. Thus, a relatively well-tolerated regime of IL-2 in dogs can induce dramatic increases in lymphocyte numbers and activation, which is associated with augmentation of their in vitro antitumor reactivity. The clinical effectiveness of this immunotherapeutic approach remains to be tested in tumor-bearing dogs where it could serve as a relevant large-animal model for immunotherapy of cancer with IL-2.

Key words: Canine – Cytotoxicity – Interleukin-2 – In vivo – Infusion – Lymphocyte

Introduction

Interleukin-2 (IL-2) is a pleiotropic lymphokine that induces a myriad of biological effects important to cancer immunotherapy. These include induction of the lymphokine-activated killer (LAK) cell phenomenon [11, 12], augmentation of antibody-dependent cellular cytotoxicity [13, 39, 48], and enhancement of macrophage-mediated tumor cytolysis [32]. These mechanisms are able to mediate regression of both primary and metastatic tumors in mice and humans [24, 33, 38, 45]. The observation that cells with LAK activity mediate potent tumor cytolysis has led to the development of clinical protocols based on in vitro culture of an individual patient's peripheral blood lymphocytes (PBL) with IL-2 so that LAK activity is generated in these PBL, which are then re-infused into the patient [9, 43, 44]. LAK cells are generally thought to be natural killer (NK) cells that have undergone IL-2 augmentation of their tumoricidal properties [41]. However, because of excessive toxicity, complex technical support, and the expense of activating NK cells outside the body with IL-2 (i.e., ex vivo), some groups have focused on induction of LAK activity in vivo via continuous infusion of IL-2 [28, 40, 52, 53]. This simplified approach for IL-2 therapy of human cancer patients has seemingly not resulted in loss of biological effectiveness [12, 52, 53]. Toxic reactions are still possible, however, that are route-, dose-, and schedule-dependent [20, 40, 53].

Because the immunological effects of prolonged continuous infusion of IL-2 in humans are greater than those of bolus dosing or short-term infusion [20, 52], we tested a low-dose IL-2 infusion protocol in dogs to determine the immunological effects on canine PBL and clinical toxicities. We evaluated a dose and schedule of intravenous IL-2 administration in normal dogs similar to that used in human cancer patients [52] in order to obtain comparative data in a canine model of IL-2 immunotherapy. Although a recent report of subcutaneous bolus human recombinant IL-2 in dogs described detectable immunological effects only in the dogs receiving the highest IL-2 dose (1.9×10^8 units/m^2) [2], our previous results suggest that marked

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lymphocyte activation could be possible in dogs given lower doses of human recombinant IL-2. This hypothesis is based on our previous findings including the following: (a) canine PBL bind human recombinant IL-2 with an affinity comparable to human PBL [16], and (b) short-term in vitro culture of canine PBL with low-dose IL-2 (i.e., a concentration of IL-2 potentially achievable in vivo without toxicity) confers augmented cytotoxic capabilities on these PBL, which are better able to kill canine NK-resistant tumor targets [17].

We have chosen to develop a canine model for cancer immunotherapy because many spontaneous neoplasms of dogs closely mimic the biology of cancers that arise in humans. Outbred dogs with spontaneous tumors have proven to be an excellent model for investigating other immunotherapeutic compounds [15, 30, 31]. The results of the current study indicate that repetitive, continuous intravenous infusion with a clinically tolerable regime of human recombinant IL-2 in the dog produces significant lymphocyte activation. Continued efforts using the clinical and immunological results from this study could form the basis for combined immunotherapy trials in tumor-bearing dogs using IL-2, tumor-reactive monoclonal antibodies, and other biomodulatory molecules to enhance effectiveness of IL-2 therapy in both canines and humans.

Materials and methods

IL-2 infusion protocol

Four healthy adult dogs (21–25 kg) received two consecutive cycles of IL-2 given by continuous intravenous infusion over 14 days. Each cycle consisted of 4 days of IL-2 treatment followed by 3 days of observation. Each dog received 3×10^6 units m^{-2} day^{-1} of human recombinant IL-2 (specific activity 1×10^7 units/mg, in sterile solution, kindly provided by Hoffmann-LaRoche Inc., Nutley, N.J.) given in 500 ml 0.9% saline with 1% pooled, heat-inactivated, filtered canine serum as a protein carrier. Units used for IL-2 are those of the National Cancer Institute Biological Response Modifiers Program. The dogs were treated by a veterinary oncologist at the Veterinary Medical Teaching Hospital of the University of Wisconsin-Madison in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Clinical monitoring

The dogs were evaluated physically for side-effects associated with the IL-2 infusion on a daily basis and arterial blood pressure (measured by the Doppler technique) was recorded three times daily. Complete blood counts were performed daily and serum biochemical profiles (Kodak Ektachem 500 Analyzer, Rochester, N.Y.) were performed weekly.

Isolation of lymphocytes

Canine PBL from the four adult dogs receiving IL-2 were obtained prior to, during, and after each cycle of the continuous 4-day in vivo infusion of IL-2. The lymphocytes were obtained from heparinized venous blood by separation over a Ficoll/Hypaque (Histopaque-1077, Sigma, St. Louis, Mo.) density gradient (specific gravity 1.077 g/ml), as described [1, 16]. The isolated cells were washed three times in Hank's balanced salt solution and were resuspended in RPMI medium supplemented with 10% *v/v* heat-inactivated fetal calf serum, 2 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES buffer, penicillin (100 U/ml), and streptomycin (100 µg/ml). The viability of the isolated PBL, as assessed by trypan blue dye exclusion, was routinely greater than 98% at the time of culture.

Immunological testing

PBL were tested in vitro to determine: (a) effects of IL-2 infusion on proliferative responses to IL-2, (b) their ability to mediate lysis of a tumor target prior to and following in vivo IL-2, and (c) immune phenotypes by flow cytometry using monoclonal antibodies prior to and following in vivo IL-2.

Lymphocyte proliferation assay. Mitogens were added to individual cultures at the indicated concentrations, and included phytohemagglutinin (PHA-P, 5 µg/ml; Sigma) and human recombinant IL-2 (100 units/ml), provided by the Hoffmann-LaRoche Company (Nutley, N.J.). Controls consisted of PBL cultured in medium.

Cultures (200 µl/well) were incubated in 96-well, flat-bottom, microtiter plates (Falcon, Oxnard, Calif.) in a 5% CO_2 atmosphere at 37° C for 2–10 days. The final concentration of PBL in each well was 1×10^6 cells/ml (2×10^5 cells/well). Each well was pulsed with 0.5 µCi [^{3}H]thymidine (specific activity 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) 8 h before termination of culture. The cells were then harvested onto fiberglass filters with a multichannel automated harvester (PhD Cell Harvester, Cambridge Technology Inc., Cambridge, Mass.). The filters were transferred to plastic scintillation vials to which 3 ml scintillation fluid (Econofluor, New England Nuclear) was added, and counted in an automated liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The data are reported as the mean radioactivities (cpm) of triplicate cultures, and the standard deviation among triplicates was less than 15%.

Tumor cells. A canine thyroid adenocarcinoma cell line (CTAC) [18, 23] (provided by Dr. John Jardine) was used as a target. This line was grown in RPMI-1640 medium supplemented with 10% fetal calf serum. CTAC is an adherent line and was detached with trypsin/EDTA (0.05% trypsin/0.53 mM EDTA for 10 min), washed with fresh medium, and used as a single-cell suspension in cytotoxicity experiments.

Cytotoxic assay. Release of chromium was used to assess lysis of target cells by effector cells [12]. Target cells were labeled with 250 µCi ^{51}Cr for 2 h at 37° C in 5% CO_2. These cells were washed in medium and 5×10^3 cells (in 50 µl) were added to round-bottom microwells containing serial dilutions of effector cells (also in 50 µl). The viability of target cells (determined by eosin exclusion) prior to their use in killing assays was greater than 98%. The concentration of effector cells was adjusted to give a final effector/target cell ratio of 100:1, 50:1, and 25:1. Cytotoxicity was measured in the presence or absence of IL-2 in 50 µl (100 units/ml), added 1 h before the target cells. The final volume of each well was adjusted to 200 µl by adding medium as necessary. All assays were done in quadruplicate. Plates were centrifuged at 500 *g* for 5 min and incubated at 37° C for 4 h or 16 h. Supernatants were then harvested with a Skatron harvesting system (Skatron, Sterling, Va.). Radiolabel released into the supernatant was measured with a gamma counter. The percentage cytotoxicity was calculated using the formula:

$$\text{cytotoxicity (\%)} = \frac{E - S}{M - S} \times 100$$

where E is the experimental radioactivity (cpm) released from target cells incubated with effectors, S is the spontaneously released radioactivity (cpm) obtained from targets incubated in medium alone, and M is the maximum radioactivity (cpm) obtained from targets lysed with a 2% cetrimide detergent solution (Sigma). ^{51}Cr release data from all three effector/target ratios were also converted to lytic units. One lytic

Table 1. Clinical toxicity of continuous IL-2 infusion in normal dogs

Toxicity	No. dogs (n = 4)	Comments
Vomiting	4	Mild, responded to symptomatic therapy (metoclopramide)
Diarrhea	4	Mucoid, with occasional blood by the 3rd day of week 1 infusion
Lethargy	4	By 2nd day of each week's infusion
Inappetence	4	Subsided by the end of 2nd week
Rigors	2	Mild
Skin erythema	1	Patchy and mild, week 1
Biochemical abnormalities	0	
Pulmonary edema	0	
Hypotension	0	
Fever	0	

All toxicities abated within 24 h of the end of IL-2 infusion, except diarrhea, which persisted for up to 72 h in two dogs

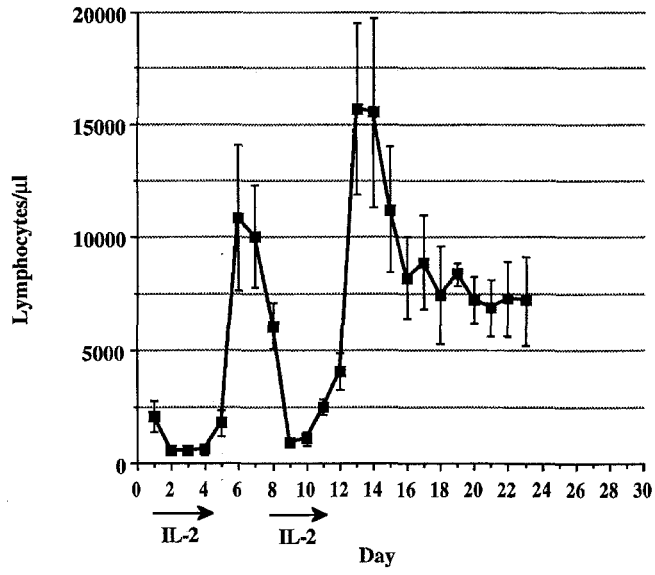

Fig. 1. Mean daily lymphocyte counts for four dogs completing two weekly cycles of interleukin-2 (IL-2) at a dose of 3×10^6 units m^{-2} day^{-1}. Each dog began a 4-day continuous infusion of IL-2 on days 1 and 8. *Error bars* represent the standard error of the mean lymphocyte counts for the four dogs

unit is defined as the number of effector cells required to cause 20% lysis of 5×10^3 target cells. Lytic units are expressed per 10^7 effector cells harvested.

Flow cytometry. Flow cytometric analyses of PBL from each dog were performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) using standard indirect immunofluorescent techniques, and gating on the lymphocytes [27]. Murine monoclonal antibody (mAb) 12.125, which is a marker for canine CD4 (helper) cells [10], mAb 4.78, a marker for canine CD8 (cytotoxic) cells [10], and mAb 8.358, a pan-T-cell marker [10], were used to label each dog's PBL. Irrelevant murine isotype control antibodies were used for each of these mAbs and included IgG1 (Becton Dickinson) for mAb 12.125 and IgM (Olympus Corporation, Lake Success, N.Y.) for mAbs 4.78 and 8.358.

Serum IL-2 concentration

The concentration of human recombinant IL-2 was measured in serum from the four dogs prior to treatment and at intervals while on IL-2 using an enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems, Minneapolis, Minn.). Conditioned medium from canine PBL activated with PHA (10 µg/ml) for 24 h and 48 h in vitro was tested for cross-reactivity of canine IL-2 using the same ELISA.

Statistical analysis. All differences were tested with paired t-tests using the SAS program.

Results

Clinical toxicity

All four dogs completed the planned 14-day course of IL-2. The observed toxicities are listed in Table 1. Gastrointestinal signs predominated consisting of vomiting and diarrhea, occasionally containing blood and mucus. Vomiting was always mild and responded to symptomatic treatment with metoclopramide. Lethargy and inappetence developed in all dogs by the second day of each week's infusion. Appetite improved towards the end of the week-2 infusion. All toxicities abated 24 h after IL-2 infusion was stopped, with the exception of diarrhea, which took up to 72 h to improve in two dogs. There were no biochemical abnormalities at any time during the study. Hypotension, edema, weight gain, tachypnea, and fever were not detected during IL-2 infusion.

Hematologic effects of treatment

Lymphocytes. Figure 1 shows the mean daily lymphocyte counts for the four dogs. There was a decrease in the mean lymphocyte count 24 h after the beginning of each cycle of IL-2, which was significant after cycle 2 ($P = 0.003$) but not after cycle 1 ($P = 0.06$). Within 24 h after each 4-day IL-2 infusion was stopped, there was a significant rebound in lymphocyte counts compared to the lymphocyte counts at the start of IL-2 for that cycle ($P = 0.02$ each cycle). The rebound lymphocytosis progressively increased after each IL-2 cycle. Compared to the pretreatment values, there was a significant ($P = 0.01$) rise in the mean lymphocyte count after two cycles of IL-2 (day 13), going from a mean of 2061/µl to 16078/µl, a 7.8-fold increase. The mean lymphocyte count declined slowly and remained elevated for at least 2 weeks. One dog's lymphocyte count was still elevated two months after IL-2 treatment. Large granular lymphocytes were occasionally present in the blood smear at the time of lymphocytosis (Fig. 2).

Eosinophils. Figure 3 shows the mean daily eosinophil counts for the four dogs. The pattern of changes in the eosinophil counts roughly paralleled that of the lympho-

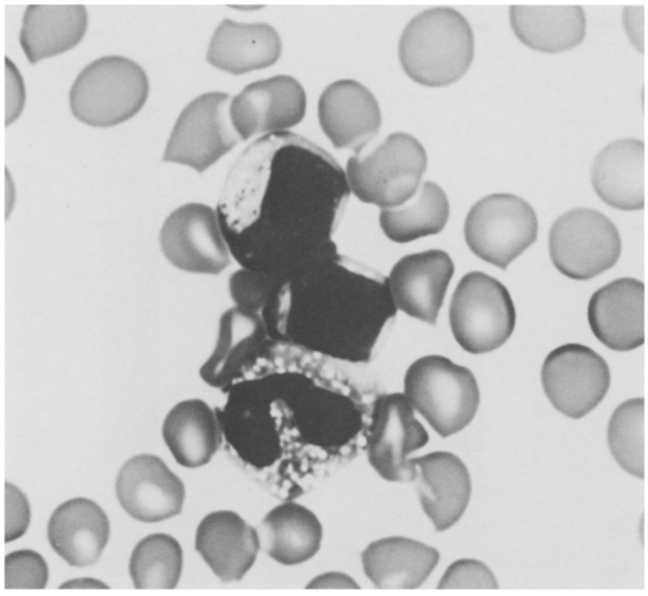

Fig. 2. Photomicrograph of nucleated cells in the peripheral blood smear from a dog after intravenous treatment with IL-2. A large granular lymphocyte is present in the top of this field and a vacuolated band eosinophil is seen at the bottom (1000× original magnification)

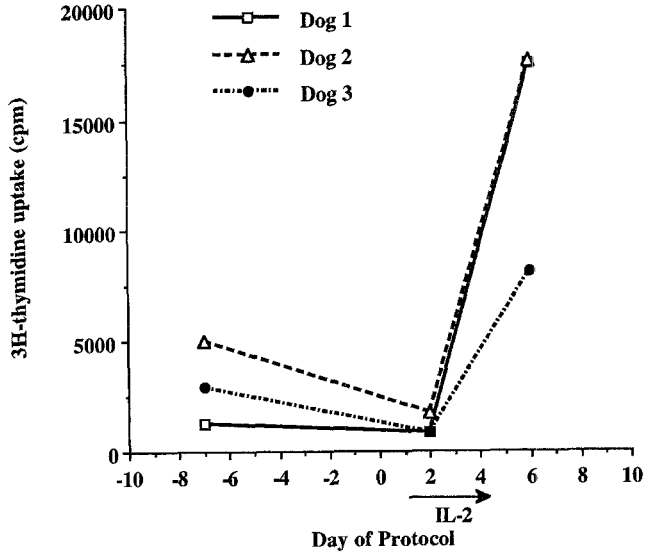

Fig. 4. Three-day in vitro proliferative response to IL-2 of peripheral blood lymphocytes obtained from three dogs prior to receiving IL-2 (3×10^6 units m^{-2} day^{-1} for 4 days), 24 h after starting IL-2 treatment (day 2), and 24 h after completing the first 4 days of continuous IL-2 infusion (day 6). The difference in proliferative response between day-6 and day-2 lymphocytes was significant ($P = 0.05$). Proliferation is measured as a function of [^{3}H]thymidine uptake and reported as the mean radioactivity (cpm) of triplicate wells

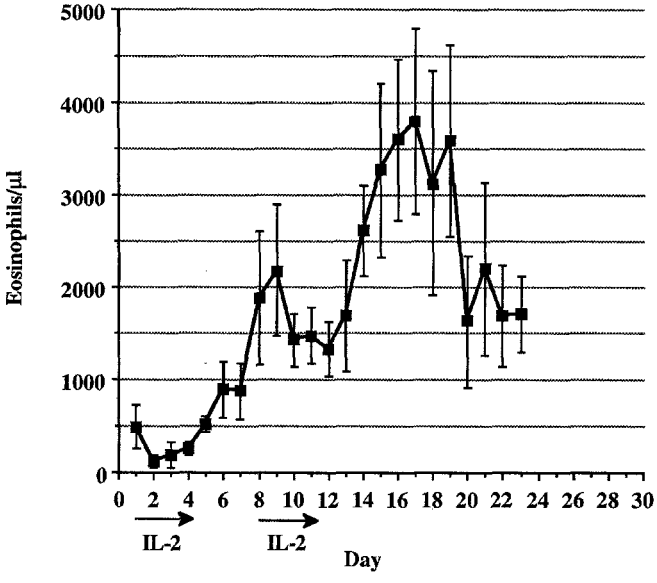

Fig. 3. Mean daily eosinophil counts for four dogs completing two weekly cycles of IL-2 at a dose of 3×10^6 units m^{-2} day^{-1}. Each dog began a 4-day continuous infusion of IL-2 on days 1 and 8. *Error bars* represent the standard error of the mean eosinophil counts for the four dogs

cytes during the 2 weeks of IL-2. However, 5–6 days after IL-2 treatment was stopped, there was a significant ($P = 0.02$ cycle 1; $P = 0.0001$ cycle 2) increase in the mean eosinophil counts compared to the eosinophil count at the start of IL-2 for that cycle (i.e., day 1 versus day 9, cycle 1; day 8 versus day 17, cycle 2). This was at the time the lymphocyte counts were decreasing. The rebound eosinophilia progressively increased after each IL-2 cycle. The magnitude of eosinophilia after cycle 2 (day 17) was significantly greater than that following cycle 1 (day 9) ($P = 0.004$). Compared to pretreatment values, there was a significant ($P = 0.002$) increase in the mean eosinophil count after two cycles of IL-2, peaking on day 17 of study. The mean eosinophil count increased 7.7-fold from the pretreatment level of 491/µl to 3794/µl after 2 weeks of IL-2 treatment. Eosinophil band forms and cytoplasmic vacuolization were observed in the blood smear during rebound eosinophilia (Fig. 2).

Lymphocyte proliferation in vitro

In vivo treatment with IL-2 modulated the in vitro lymphocyte proliferative response to IL-2 (Figs. 4, 5). Resting PBL obtained from control dogs or from dogs prior to IL-2 proliferated in response to IL-2. This response was first detectable on day 6 of culture (Fig. 5) [16]. PBL obtained following 2 weeks of IL-2 demonstrated a markedly enhanced response to IL-2. This response was detectable by day 2 of culture and the peak response was three times that seen with lymphocytes obtained prior to therapy. Lymphocytes obtained 24 h after completion of the first IL-2 cycle (day 6) showed significantly enhanced 3-day proliferative responses to IL-2 compared to lymphocytes obtained after 24 h of IL-2 infusion ($P = 0.05$). Compared to the pretreatment PBL, in vitro mitogenic responses to IL-2 of PBL obtained after completing 2 weeks of in vivo treatment with IL-2 occurred earlier, progressed faster, and stimulated more cells relative to the proliferative response of pretreatment cells to IL-2. The proliferative response to the T-cell mitogen PHA was not altered by IL-2 treatment (Fig. 5). Because of an incubator failure, results are avail-

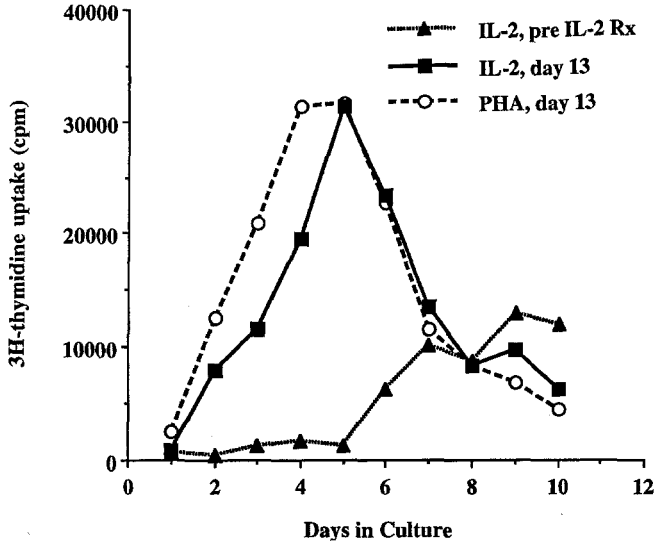

Fig. 5. Comparison of peripheral blood lymphocyte (PBL) in vitro proliferative responses from a dog prior to (*pre-IL-2 Rx*) and after two weekly cycles (*day 13*) of IL-2 infusion. IL-2 (100 units/ml) and phytohemagglutinin (PHA; 5 μg/ml) were included with some PBL cultures as indicated. PBL grown in medium alone did not proliferate (not shown). Proliferation is measured as a function of [^{3}H]thymidine uptake and reported as the mean radioactivity (cpm) of triplicate wells

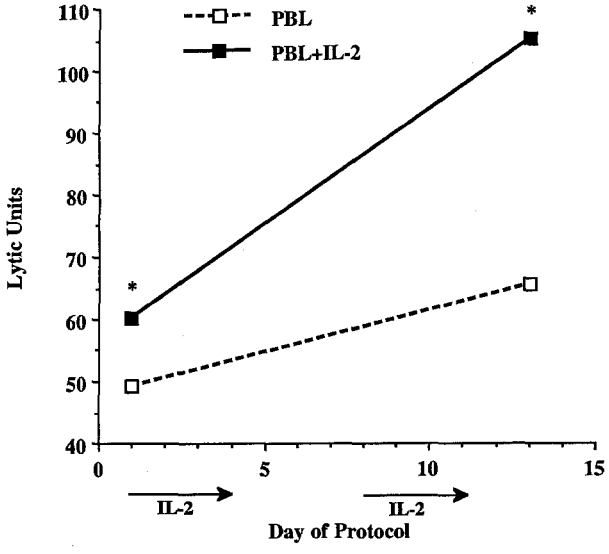

Fig. 6. Lytic activity of peripheral blood lymphocytes (PBL) from three dogs obtained prior to starting IL-2 (3×10^6 units m^{-2} day^{-1} by continuous infusion for 4 days) and 24 h after completing two weekly cycles of treatment. Effector cells were incubated for 1 h with medium or IL-2 prior to addition of CTAC target cells. * $P = 0.01$

able on only three of the four dogs for the proliferation and cytotoxicity assays. The cell count and proliferation studies suggest that in vivo IL-2 initially caused cells responsive to IL-2 in vitro to leave the circulation. Following the IL-2 treatment, cells responsive to IL-2 returned to the circulation as shown by the in vitro IL-2-proliferative response in post-treatment PBL. These lymphocytes responded sooner, and the response was of greater magnitude than observed in pretreatment PBL.

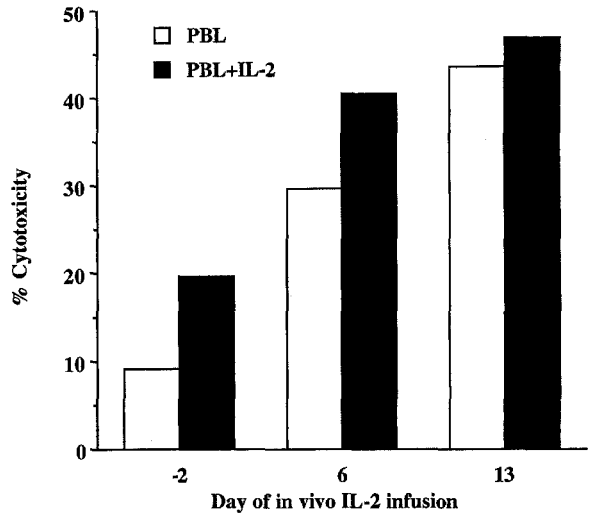

Fig. 7. Cumulative effect of IL-2 treatment (3×10^6 units m^{-2} day^{-1} by continuous infusion) on direct cytotoxicity (16-h ^{51}Cr release) of CTAC targets by PBL from a dog completing IL-2 treatment given on days 1–4 and 8–11. Each *bar* represents direct cytotoxicity at an effector-to-target cell ratio of 100:1. Effector cells were incubated for 1 h with medium or IL-2 prior to addition of CTAC target cells

Effect of in vivo IL-2 on cell mediated tumor lysis by PBL in vitro

In a 16-h ^{51}Cr-release assay, the direct cytotoxicity against CTAC target cells mediated by post-treatment PBL (from three dogs), obtained 24 h after two cycles of in vivo IL-2 treatment, was greater than that mediated by pretreatment PBL ($33.4 \pm 4.3\%$ versus $25.8 \pm 10.4\%$, $P = 0.4$). Adding IL-2 during the assay augmented direct cytotoxicity by post-treatment PBL more than that by pretreatment PBL ($46.8 \pm 5.0\%$ versus $30.8 \pm 9.9\%$, $P = 0.08$). These differences were not significant. When the expansion of lytic cells is taken into consideration, and the cytotoxic potential or lytic units per ml of blood determined, there was a marked enhancement with treatment. As a mean value, pretreatment PBL mediated 49.2 lytic units compared to post-treatment PBL, which mediated 65.7 lytic units (Fig. 6). The addition of IL-2 during the cytotoxicity assay facilitated greater augmentation of cytotoxicity against the CTAC target by post-treatment PBL, mediating 105.3 ± 18.5 lytic units compared to 60.2 ± 21.3 for pretreatment PBL, which was significant ($P = 0.01$) (Fig. 6). Therefore, the in vitro exposure to IL-2 enhanced the cytotoxicity of post-treatment PBL more than that of pretreatment PBL. Thus, the in vivo IL-2 treatment activated canine PBL, rendering them more sensitive in vitro to the IL-2 cytotoxic augmenting effects against the CTAC target. In vivo enhancement of lymphocyte cytotoxicity by IL-2 infusion was cumulative. The greatest augmentation in cytotoxicity was detected 24 h after the full 2-week course had been completed. Representative data from a single dog are shown in Fig. 7.

Table 2. Lymphocyte immunophenotypes of dogs receiving continuous IL-2 infusion

	Pan-T	CD4	CD8
A. Percentage			
Pre IL-2 ($n = 4$)	78±4.1	46±1.6	26±4.0
Day 2 ($n = 2$)	85±2.0	45±2.5	38±1.5
Day 5 ($n = 3$)	89±2.3	61±1.2	23±4.0
Day 13 ($n = 4$)	91±2.2	60±2.4	16±1.5
B. Absolute No/μl			
Pre IL-2	1608± 517	948± 305	536±172
Day 2	468± 65	248± 35	209± 29
Day 6	9654±2860	6617±1960	2495±739
Day 13	14290±3460	9422±2281	2512±608

Lymphocyte phenotype

The relative and absolute number of circulating PBL expressing T-cell markers detected by monoclonal antibodies are shown in Table 2. Twenty-four hours after IL-2 treatment was initiated, there was a drop in the number of lymphocytes expressing each marker, corresponding to the decrease in total lymphocyte count on that day. The relative distribution of cells positive for T-cell markers remained essentially unchanged, however. Twenty-four hours after the end of the second week of IL-2 infusion, the absolute number of cells expressing CD4 had increased 10-fold ($P = 0.02$) and CD8-positive cells increased 5-fold ($P = 0.02$) over pretreatment values. Prior to IL-2 treatment, the total number of cells expressing CD4 and CD8 antigens was less than the number of lymphocytes positive for the pan-T-cell antibody; 6% (96/μl) of the pan-T-positive cells were CD4- and CD8-negative. Twenty-four h after completing the second week of IL-2, 15% (2356/μl) of pan-T-positive cells were CD4- and CD8-negative. This represents a 25% increase in the absolute number of pan-T^+, $CD4^-$, $CD8^-$ cells.

Serum IL-2 concentration

No human recombinant IL-2 was detectable in the serum from any dog prior to infusion. After 3 days of continuous IL-2 infusion, the mean serum concentration of human recombinant IL-2 in the four dogs was 9.5 units/ml (range 6.5–11.6). In the second week, the mean serum IL-2 concentration after 3 days of IL-2 infusion was 5.7 units/ml (range 1.8–13.1). Twenty-four h after completing the second course of IL-2, serum IL-2 concentrations were 0.5 units/ml or less in three dogs and 6 units/ml in the remaining dog. Canine IL-2, present in PHA-stimulated canine-PBL-conditioned medium [16] was not recognized by this assay.

Discussion

In vivo treatment of humans with recombinant IL-2 activates a population of immune cells with antitumor activity [12, 52]. Additional biological effects of IL-2 include stimulation of lymphocyte proliferation in vitro and in vivo [7, 36], and induction of release of other cytokines by stimulated immune cells [6, 8, 19, 42]. The major problem associated with in vivo IL-2 therapy has been toxicity associated with high-dose regimens requiring intensive-care monitoring of these patients [45]. Continuous infusion of IL-2, given at lower doses, has proved effective at reducing toxicity while maintaining biological activity [21, 52]. This approach overcomes the problem of the short half-life of IL-2 in vivo and activates lymphocytes more effectively than intravenous bolus dosing of IL-2 [21]. In this study, we investigated comparative aspects of a continuous IL-2 infusion protocol in healthy dogs (3×10^6 units m^{-2} day^{-1} intravenously for 4 days, repeated twice). We found that marked activation of immune effector cells developed following IL-2 treatment in these dogs.

Two consecutive weekly cycles of 4-day continuous intravenous IL-2 administration was tolerated in dogs at the dose investigated. Gastrointestinal effects were the clinical toxicities of greatest significance, consisting of mucoid diarrhea and mild vomiting. Similar signs have been seen in human patients treated with IL-2 at a dose and schedule identical to that used in this study [52]. Cain et al. reported diarrhea in dogs receiving IL-2 but only when at least 1.9×10^7 units/m^2 were given subcutaneously once daily for 5 consecutive days [2]. In the dogs of this report, diarrhea was problematic but not dose-limiting. We did not consider it severe enough to preclude investigating ambulatory, outpatient intravenous IL-2 treatment, given with a portable pump in dogs, at a later date.

There were marked increases in the number of activated IL-2-dependent lymphocytes in the peripheral blood following treatment with continuous infusion IL-2. The pattern of circulating lymphocyte responses to IL-2 paralleled that observed in humans receiving repetitive cycles of continuous IL-2 therapy [52]. Twenty-four hours after each cycle of IL-2 began, the peripheral blood lymphocyte counts declined significantly and remained low (relative to the lymphocyte count at the beginning of that IL-2 cycle) during the period of IL-2 infusion. Rebound lymphocytosis developed in all dogs 24 h after stopping IL-2 and tended to increase progressively after the second cycle of IL-2, though not significantly. This suggests that IL-2-responsive cells leave the circulation acutely when IL-2 infusion is begun. The emergence of large numbers of lymphocytes into the peripheral circulation following IL-2 treatment suggests that IL-2 induced expansion of the lymphoid mass at extra-vascular sites. That the magnitude of lymphocytosis was progressive implies that the first cycle of IL-2 expanded the number of IL-2-responsive cells, contributing to the incremental increases in lymphocyte numbers observed after the second IL-2 cycle. IL-2 up-regulates expression of IL-2 receptors on lymphocytes in vitro [51] and this relationship could explain expansion of the IL-2-responsive lymphocyte pool with subsequent IL-2 treatments. It is uncertain if additional cycles of IL-2 would have resulted in further increases in the peripheral lymphocyte counts. In comparison to the report by Cain et al., who gave dogs IL-2 subcutaneously, lymphocytosis was detected only in dogs receiving 1.9×10^8 units IL-2 m^{-2} day^{-1}, a dosage two orders of magnitude greater than that ad-

ministered to the dogs described in this report [2]. In that study, lymphocytosis was not as pronounced or prolonged as that seen in the current investigation. Seemingly, in vivo IL-2 administration by continuous infusion is more effective at expanding lymphoid mass than the subcutaneous route in dogs.

Other evidence that IL-2-responsive lymphocytes leave the circulation during IL-2 infusion and return with up-regulated expression of IL-2 receptors after IL-2 treatment is stopped is seen in the in vitro lymphocyte proliferation data. IL-2 is mitogenic for lymphocytes that express IL-2 receptors [50]. In the proliferation assay, lymphocytes cultured with IL-2 that were obtained on the second day of IL-2 infusion when the dogs were lymphopenic, incorporated less [^{3}H]thymidine compared to pretreatment lymphocytes. Lymphocytes obtained during the period of lymphocytosis (24 h after the end of the first cycle of IL-2) incorporated greater amounts of [^{3}H]thymidine into DNA and responded to IL-2 earlier during in vitro culture when compared to pretreatment lymphocytes. The magnitude and kinetics of this response paralleled values seen following lymphocyte stimulation with PHA (Fig. 5). As the same number of cells were always seeded into the microtiter wells, the likely explanation for the greater [^{3}H]thymidine uptake in post-treatment lymphocytes is that more of these cells were induced to proliferate by IL-2. Because IL-2-driven mitogenic responses are triggered by IL-2 binding to its receptor [50], the IL-2 treatment likely up-regulated expression of the IL-2 receptor complex on these PBL in vivo. As all known biological effects of IL-2 are mediated through the IL-2 receptor complex, a larger proportion of post-treatment lymphocytes must have expressed IL-2 receptors. Expression of the IL-2 receptor on lymphocytes is indicative of cellular activation [26, 49].

In the cytotoxicity assays, post-treatment lymphocytes mediated enhanced lytic activity against a canine tumor target. Exposure of post-treatment lymphocytes to IL-2 during the assay augmented tumor cytotoxicity more than that of pretreatment lymphocytes. This suggests that the in vivo treatment with IL-2 activated a population of canine lymphocytes that acquired a relative "dependence" on IL-2 for maximizing their cytotoxic function. This result is consistent with our prior in vitro findings that inclusion of IL-2 with canine lymphocytes previously cultured with IL-2 significantly enhances their LAK activity against some tumor targets [17]. The same phenomenon has been described for in vitro LAK activity mediated by lymphocytes from human cancer patients treated with IL-2 in vivo [14, 52]. Thus, several lines of evidence indicate that the expanded lymphoid mass resulting from in vivo IL-2 treatment in dogs is dependent on IL-2 for cytotoxic and proliferative function. It is unclear from this study if additional cycles of IL-2 would continue to have a cumulative incremental effect on cytotoxicity, as reported in humans [52].

We were interested in determining the effects of in vivo IL-2 on the composition of the canine lymphocyte immunophenotype. In man, IL-2 expands lymphocytes expressing several markers including pan-T (CD3), NK (CD56, Leu11, CD16) and the α subunit of the IL-2 receptor (p55, Tac) (CD25), a marker of lymphocyte activation [26, 52, 57]. Limitations on the availability of monoclonal antibodies that recognize comparable epitopes on canine lymphocytes precluded efforts to obtain similar information in the dog. We found no cross-reactivity of commercially available murine monoclonal antibodies (Becton Dickinson, Mountain View, Calif.) for human CD3, CD56, CD16, or CD25 with the canine lymphocytes of interest (data not shown). We did employ several recently described murine monoclonal antibodies that are specific markers of canine pan-T cells, canine helper T cells (CD4), and canine cytotoxic T cells (CD8). IL-2 infusion induced significant increases in the lymphocytes bearing these markers.

We observed that the number of pan-T-positive lymphocytes was greater than the total number of CD4-positive and CD8-positive lymphocytes. This population of pan-T-positive, CD4-negative, CD8-negative lymphocytes increased by 25% following 2 weeks of IL-2 infusion in these dogs. While we could not specifically determine the complete immunophenotype of these cells, it is possible they are NK cells. This would be compatible with what is known about the effects of IL-2 in other species and our own data indicating augmentation of spontaneous lymphocyte antitumor cytotoxicity following IL-2 treatment. In humans and mice, the CD3 chain with most antigenicity is the ε chain [25, 54, 55], and monoclonal antibodies against ε usually induce mitosis in human T cells [4, 34]. The epitope recognized by the canine pan-T-cell antibody we used is not known, but it is unlikely to be CD3 ε as this antibody did not induce canine PBL to proliferate (data not shown). That canine NK cells could be positive for a non-CD3 pan-T-cell antibody is not inconsistent with the previous observation that canine NK and T cells share a common lineage [29].

Significant elevations in the peripheral blood eosinophil counts were seen in all dogs following IL-2 treatment. This finding is consistent with other reports of IL-2 administration in the dog [2, 3] and in humans [8, 21, 22]. In our study, peak eosinophil counts occurred 5–6 days following the IL-2 infusion and 4–5 days after peak lymphocyte counts. There is evidence that IL-2 induces secretion of IL-5 by human T cells, which in turn is stimulatory to eosinophilopoiesis [6]. While we made no attempt to define this relationship as the mechanism responsible for eosinophilia in the dogs of this study, it is possible that a similar pathway could account for these hematological findings. The delay in eosinophilia compared to lymphocytosis could be due to the time needed for IL-2 to induce secretion of IL-5 in (still to be described) canine T helper 2 cells [5, 37]. An alternative explanation is that IL-2 had a direct effect on eosinophil precursors bearing IL-2 receptors. Future studies will need to be done to address this issue more fully. In conjunction with eosinophilia, vacuolated eosinophils were frequently seen in the blood smears. Vacuolization is considered a sign of eosinophil activation and is associated with a less dense population of eosinophils when separated by density gradient [56]. In humans, hypodense eosinophils appear in the circulation during IL-2 therapy [46] and are more cytotoxic than eosinophils of greater density [56]. While we did not specifically assay canine eosinophil cytotoxicity, peripheral blood eosinophilia and vacuolization

suggest that in vivo IL-2 treatment activates canine eosinophils as well as lymphocytes.

Defining the clinical and immunological effects of IL-2 in the dog, as shown here, complement this approach to cancer immunotherapy in mice and humans. The dog is becoming an increasingly important spontaneous large-animal metastasis model for cancer immunotherapy [30, 35, 47], and the results of this study provide the basis for follow-up trials of IL-2, combined with other biomodulatory molecules, in dogs with spontaneous malignancies that could benefit canine and human cancer patients. Through these efforts, it may eventually be possible to examine new strategies for IL-2-based therapy in this model that would reduce treatment risks for human patients.

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