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Original Article

Effects of Administration of Recombinant Human Interleukin-2 in Dogs

G. R. Cain[1], T. Kawakami[1], N. Taylor[1] and R. Champlin[2]

[1]Laboratory for Energy Related Health Research, University of California, Davis, and [2]Transplantation Biomedical Unit, University of California, Los Angeles, California, USA

Abstract. The clinical and immunohaeamatological effects of recombinant humen interleukin-2 (rhIL-2) administration were evaluated in normal dogs. Three groups of three dogs per group were administered rhIL-2 subcutaneously at a dose of 6×10^4 IU, 6×10^5 IU, or 6×10^6 IU/kg once daily for five consecutive days. Toxic clinical signs were limited primarily to diarrhoea, the severity of which, was dose dependent, with resolution within 7 days of the last rhIL-2 injection. Marked circulating eosinophilia occurred in dogs of the two highest dose groups and transient rise in blood lymphocyte numbers occurred in dogs given the highest dose of rhIL-2. The most significant immunological effects were elevated in vitro conA and pokeweed mitogen-stimulated lymphocyte blastogenic responsiveness in the highest dose group and dose-dependent elevation of antigen-specific antibody (IgG and IgM) production. Peak relative antibody production was markedly elevated, as compared to controls, in dogs administered 6×10^5 IU, 6×10^6 IU rhIL-2/kg.

Keywords. Dogs; Recombinant human interleukin-2

Introduction

Interleukin-2 (IL-2) is a lymphokine produced by antigen or mitogen activated T lymphocytes and natural killer cells (Morgan et al. 1976; Scala et al. 1986). Initially it was described as a growth factor which permitted indefinite in vitro propagation of T lymphocytes but has subsequently been demonstrated to support several other immune functions including the growth of cytotoxic T cells and natural killer cells (Gilles and Smith 1977; Grimm et al. 1982; Flomenberg et al. 1983). The immunoregulatory role of IL-2 is further demonstrated by its ability to stimulate the induction and growth of lymphokine-activated killer (LAK) cells, induce gamma-interferon production, and drive B-cell proliferation and immunoglobulin production (Farrar et al. 1981; Kauttab and Maisel 1987; Waldman et al. 1984; Zubler et al. 1984; Mitchel et al. 1991).

Due to the importance of IL-2 regulation of immune function, there is interest in its potential effectiveness in vivo for treatment of immunodeficiency. The ability of IL-2 to induce tumoricidal activity of lymphocytes and mediate regression of neoplasms in mice has also led to efforts to evaluate its application in cancer therapy (Kedar et al. 1984). The parenteral administration of large amounts of IL-2 in rodents results in reduction of established neoplasms and regression of metastatic lesions (Rosenberg et al. 1985a). In initial human clinical studies adoptive immunotherapy utilising IL-2 stimulated lymphocytes and parenteral administration of IL-2 had impressive antitumour effects (Herberman 1987; Mier 1987). The effectiveness of IL-2 administration is dependent on dose and duration of treatment (Ettinghausen and Rosenberg 1986). Unfortunately, although successful treatment of several types of neoplasms has occurred with administration of IL-2, significant clinical side effects limit its use (Rosenberg et al. 1985b).

The cloning of the human IL-2 gene has allowed for economic production of large quantities of IL-2 for clinical use. The in vitro and in vivo immunomodulating effects of IL-2 in mice, in vitro enhanced lymphocyte blastogenesis in cattle, pigs, horses, sheep, dogs and cats, and enhanced in vitro tumour cytotoxicity in dogs are indicative of cross-species activity of human IL-2

Correspondence and offprint requests to: Gary R. Cain, Syntex Discovery Research, 3401 Hillview Avenue, Palo Alto, CA 94303, USA.

(Stott et al. 1986; Fenwick et al. 1988; Jardine et al. 1989; Mitchel et al. 1991; Raskin et al. 1991).

In this study, we determined the acute clinical toxicity and lymphohaematological effects of systemic administration of recombinant human IL2 (rhIL-2) in normal dogs. This was done in an attempt to determine the potential application of rhIL-2 for treatment of canine cancer patients or experimentally in canine models of immunology and bone marrow transplantation.

Materials and Methods

Human recombinant interleukin-2 was obtained in lyophilised form from Cetus Corporation (Emeryville, CA). The human IL-2 gene was isolated from the Jurkat T cell leukaemia cell line and introduced into *Escherichia coli* as described by Wang et al. (1984). Interleukin-2 was purified to 99% homogeneity by sodium dodecylsulphate polyacrylamide gel electrophoresis. Endotoxin activity was 0.0056 ng/mg as determined by limulus amoebocyte lysate assay. Each vial containing 1.8 mg rhIL-2 (18.0×10^6 IU/kg) was reconstituted with water for injection to appropriate dilutions as required for the designated treatment groups.

Twelve beagles, 6–10 months old with an identical history of immunisation, housing, and diet were utilised. A single rhIL-2 dose or excipient was administered subcutaneously on day 1 to determine the clinically acceptable dose range for later sequential rhIL-2 injections administered on days 8–12. Dogs were assigned to one of four experimental groups with three dogs per group and each dog identified by ear tattoo. One littermate was assigned per group. Dosage levels were assigned as follows: group 1, 6×10^4 IU/kg rhIL-2 administered subcutaneously; group 2, 6×10^5 IU/kg; group 3, 6×10^6 IU/kg; control group, excipient only.

Complete blood counts (CBCs) were obtained prior to the single clinical range-finding injection, and on day 8, 8h after the initial daily injection (designated day 8.3), and on days 9, 11, 15, 27 and 36. Serum biochemical profiles were obtained prior to initial rhIL-2 injection and on day 36 at the conclusion of the study.

In vitro lymphoblastogenic responses to lectins concanavalin A (conA) (Calbiochem, San Diego, CA), phytohaemagglutinin A (PHA) (Calbiochem), and pokeweed mitogen (PKW) (Difco Laboratories, Detroit, MI) were determined using a whole blood microassay on days 0, 14, and 33 (Shifrine et al. 1978). Briefly, blood was collected in 100 U of preservative-free heparin/ml of blood. Whole blood (50 µl) was added to 900 µl of complete medium and optimal mitogen concentrations of conA or PKW (20 µg/ml and 1 µg/ml, respectively) were added. Responses to PHA included activity after optimal stimulating concentrations (10 µg/ml) and suboptimal concentrations of 1, 2.5 and 5 µg/ml in order to determine if rhIL-2 administration increased T lymphocyte reactivity in suboptimal in vitro stimulating concentrations. Cells were pipetted into wells of flat-bottomed microtitration plates, incubated at 37 °C in a humidified 5% CO_2 atmostphere for 5 days, labelled with radioisotope (tritium), harvested, and counted. Stimulation indices were determined for each lectin concentration for each dose group at each examination date by dividing the radioisotope counts of wells from stimulated lymphocytes of each dog by the counts of unstimulated lymphocytes from the same dog. Mean stimulation indices were determined from the three dogs at each lectin dose level and date.

Dogs were immunised with keyhole limpet haemocyanin (KLH) (Behring Diagostics, La Jolla, CA), 1 mg/kg subcutaneously on days 1, 8, and 25 to determine effects of rhIL-2 injections on specific antibody response. Relative antibody concentrations were determined in control and experimental dogs by enzyme-linked immunosorbent assay (Voller et al. 1979). Briefly, KLH in sodium carbonate/biccarbonate coating buffer (pH 9.2) was coated onto 96-well microtest plates. Sera from pre- and postsensitised dogs were collected aseptically and stored at 30 °C. All sera were diluted 1:1000 to 1:4000 in phosphate-buffered saline, pH 7.2 (PBS) supplemented with 1% bovine serum albumin and 0.01% NaN_3. Wash solution which was used to rinse after each reaction consisted of PBS with 0.05% Tween 20 (Sigma Chemical Co., St Louis, MO). Diluted serum was dispensed in triplicate onto washed KLH-coated plates and incubated for 20h at 4 °C. Serum IgG and IgM anti-KLH antibodies were detected with biotinated goat anti-dog IgG or IgM (Kirkegaard and Perry Lab Inc., Gaithersberg, MD) followed by avidin-peroxidase. Peroxidase substrate contained 1mg/ml *o*-phenylenediamine 2HCl (Sigma) in 0.1 M-sodium citrate/citric acid buffer, pH 5.0, and 0.015% v/v H_2O_2. Serum and peroxidase substrate were dispensed at 100 µl/well and other reagents were dispensed at 50 µl/well. The reaction colour was read photometrically at maximal absorption wavelength (492 nm) in a spectrophotometric plate reader. Absorption values were recorded and plotted graphically as absorbance (A).

Complete physical examinations were performed prior to rhIL-2 injection and 8h after rhIL-2 injection. Single daily physical examinations were performed on days in which rhIL-2 was not administered. Rectal temperature, general physical condition, and condition of injection sites were evaluated and recorded.

The Mann–Whitney U probability test was used to compare the effects of rhIL-2 administration on lymphocyte blastogenesis and anti-KLH antobody response (Zar 1984).

Results

Clinical Response

Group 1. No fevers were recorded in dogs from group 1. Two dogs had minor pain elicited after initial injection of rhIL-2. One dog had a 1 cm diameter firm subcutaneous swelling at an injection site which resolved within 7 days. None of the dogs had diarrhoea (Table 1).

Table 1. Clinical abnormalities observed in three rhIL-2 dose groups and control dogs. Number of dogs affected per treatment group. Three dogs in each group

	Group[a]			
	1	2	3	4
Transient fever	0	1	0	0
Pain at injection	2	1	0	0
Swelling at injection site	1	2	3	0
Diarrhoea	0	3	3	0

rhIL-2, recombinant human interleukin 2.
[a]Group 1: 6×10^4 IU rhIL-2 subcutanteously; group 2: 6×10^5 IU rhIL-2; group 3: 6×10^6 IU rhIL-2; group 4: excipient control.

Group 2. One dog had transient fever (39.6 °C) after initial rhIL-2 injection. Rectal temperature was normal after 24h. One dog elicited a painful response after initial rhIL-2 injection and two dogs had mild non-painful subcutaneous induration (1 cm diameter) at the initial injection site. The swelling sites resolved within 7 days. All three dogs had mild to moderate mucoid diarrhoea which started on the days 3–5 of daily rhIL-2 injections. Diarrhoea persisted for 1 to 2 days.

Group 3. No fevers were recorded in any dog. Non-painful subcutaneous swelling (1–3 cm diameter) at a single rhIL-2 injection site was palpated in all three dogs. Subcutaneous swellings were resolved within 7 days. All three dogs had mild to marked mucoid to fluid diarrhoea for 4–6 days beginning on the third to fourth day of daily rhIL-2 injections. One dog had melena for 4 days. All dogs were clinically normal within 7 days after the last injection of rhIL-2.

Group 4. No abnormal clinical signs were observed.

Serum Biochemical Profiles. No clincally significant alterations in serum biochemical profiles were observed in any treatment group or control dogs.

Haematological Response

Neutrophils. All treatment groups had a transient increase in neutrophil count 8h after the first of five daily rhIL-2 injections; however, a similar rise in neutrophil count was also present in the control group (Fig. 1). Neutrophil counts in the three treatment groups thereafter generally parallelled those of the control group.

Lymphocytes. Compared to controls, there was a transient decrease in circulating numbers of lymphocytes in dogs 8h after the first of five daily rhIL-2 injections. A marked rebound above baseline numbers (350%) occurred in mean lymphocyte numbers in group 3 dogs at day 15. Lymphocyte counts normalised at the next observation point (day 27) in group 3 dogs (Fig. 2).

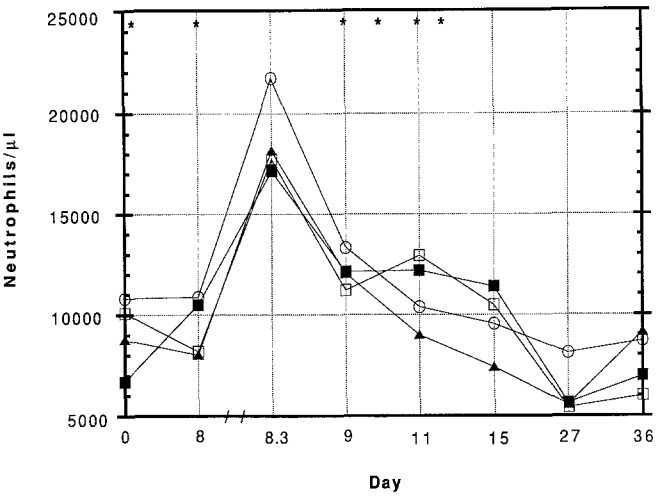

Fig. 1. Mean circulating neutrophil response in dogs administered subcutaneous recombinant human interleukin-2 or excipient (groups 1–3; 6×10^4, 6×10^5, or 6×10^6 IU/day, respectively). No marked changes were seen in dogs administered recombinant human interleukin-2 as compared to control dogs. *, day of administration of recombinant human interleukin-2; ▲, control group; o, group 1; □, group 2; ■, group 3.

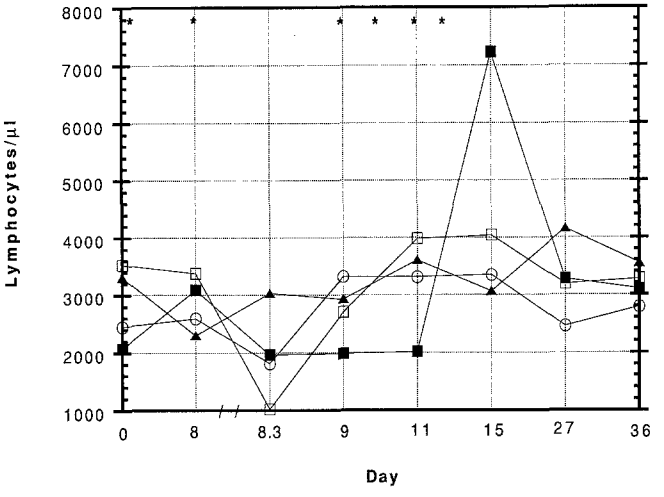

Fig. 2. Mean circulating lymphocyte response in dogs administered subcutaneous recombinant human interleukin-2 or excipient (groups 1–3; 6×10^4, 6×10^5, or 6×10^6 IU/day, respectively). Marked increase in lymphocyte count compared to control dogs was seen at day 15 for dogs in group 3. *, day of administration of recombinant human interleukin-2; ▲, control group; o, group 1; □, group 2; ■, group 3.

Eosinophils. Marked increase in eosinophil count occurred predominantly in dogs from groups 2 and 3. Eosinophil counts peaked at day 15 (mean 6×10^3/µl, group 2; 5.4×10^3µl, group 3). Eosinophil counts were back to baseline numbers by day 27 (Fig. 3).

Red Blood Cells. Red blood cell counts remained within normal range for all dose groups throughout the observation period (mean range: 6.02–6.95 $\times$ 10^6 cells/µl).

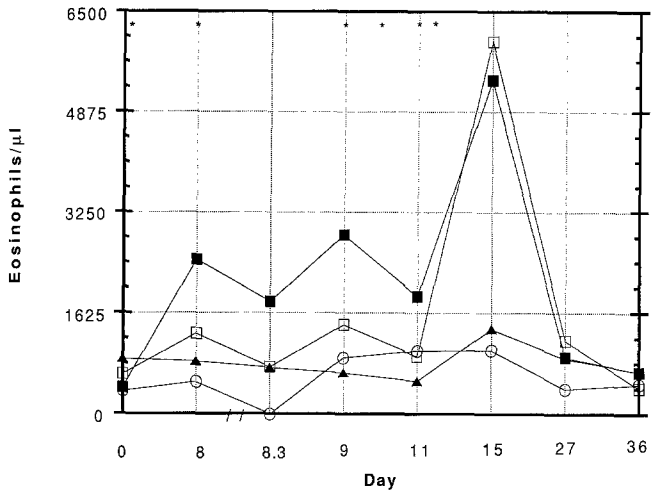

Fig. 3. Mean circulating eosinophil response in dogs administered subcutaneous recombinant human interleukin-2 or excipient (groups 1–3; 6×10^4, 6×10^5, or 6×10^6 IU/day, respectively). Marked increase in eosinophil count occurred in groups 2 and 3 at day 15 compared to control dogs seen at day 15. ▲, control group; o, group 1; □, group 2; ■, group 3.

Platelet Counts. Platelet counts remained in the normal range for all dose groups within the treatment and observation periods of 35 days (mean platelet count = 252–487 $\times 10^3/\mu l$).

Immunological Responses

Lymphocyte Blastogenesis. Spontaneous lymphocyte blastogenesis was not markedly affected in any group of dogs administered rhIL-2. The only significant or markedly altered in vitro lectin-induced lymphoid blastogenic response occurred in group 3 at day 33 for the lectins conA and PKW ($p = 0.04$ and 0.08, respectively) (Figs. 4 and 5). Blastogenic responses were not markedly altered from control values in groups 1 and 2 in response to mitogens conA and PKW. No significant differences were observed in the response of any treatment groups to PHA at optimum or suboptimal in vitro stimulating concentrations.

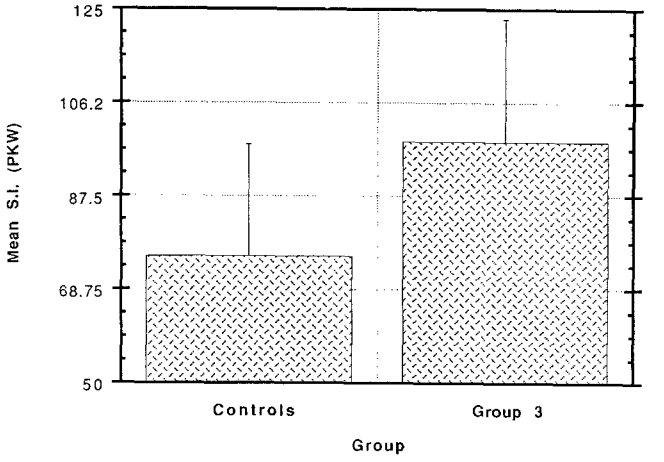

Fig. 5. Lymphocyte blastogenic response (mean stimulation index, pokeweed (PKW) in dogs administered 6×10^6 IU recombinant human interleukin-2/day (group 3) compared to values determined in control dogs that received excipient only. Increased blastogenic response compared to control dogs was observed at day 33 ($p = 0.08$). Error bar = SEM. S.I. = stimulation index (determined by dividing the radioisotope counts from wells of stimulated lymphocytes of each dog by the counts from wells of unstimulated lymphocytes from the same dog). The mean S.I. was determined from three dogs per dose group.

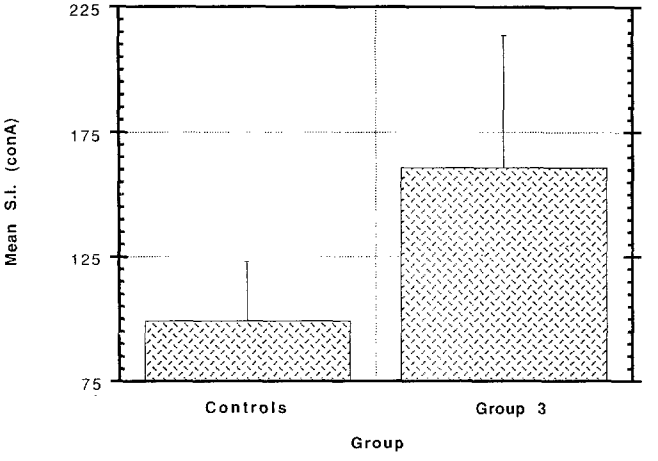

Fig. 4. Lymphocyte blastogenic response (mean stimulation index, concanavalin A (conA) in dogs administered 6×10^6 IU recombinant human interleukin-2 (group 3) compared to values determined in control dogs that received excipient only. Increased blastogenic response compared to control dogs was observed at day 33 ($p = 0.04$). Error bar = SEM. S.I. = stimulation index (determined by dividing the radioisotope counts from wells of stimulated lymphocytes of each dog by the counts from wells of unstimulated lymphocytes from the same dog). The mean S.I. was determined from three dogs per dose group.

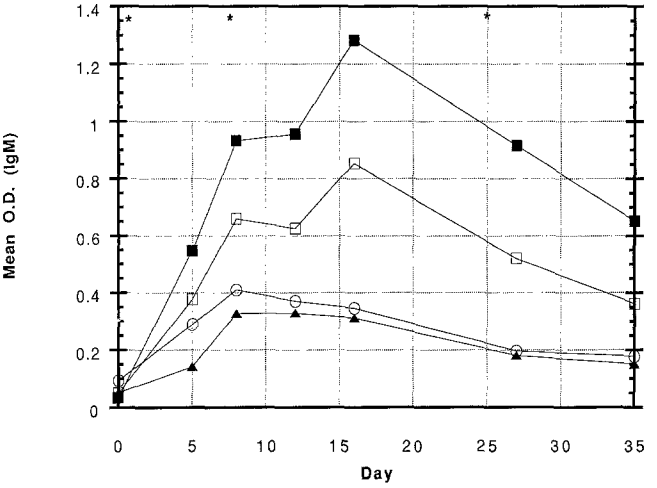

Fig. 6. Anti-keyhole limpet haemocyanin (KLH) IgM antibody response to recombinant human interleukin-2 administration. A marked increase in relative antibody levels compared to control dogs was observed at day 16 for dogs from groups 2 and 3 (6×10^5 and 6×10^6 IU/day, respectively; $p = 0.08$). O.D., optical density (absorption reading) recording of the spectrophotometric reader; *, day of administration of KLH; ▲, control group; o, group 1; □, group 2; ■ group 3.

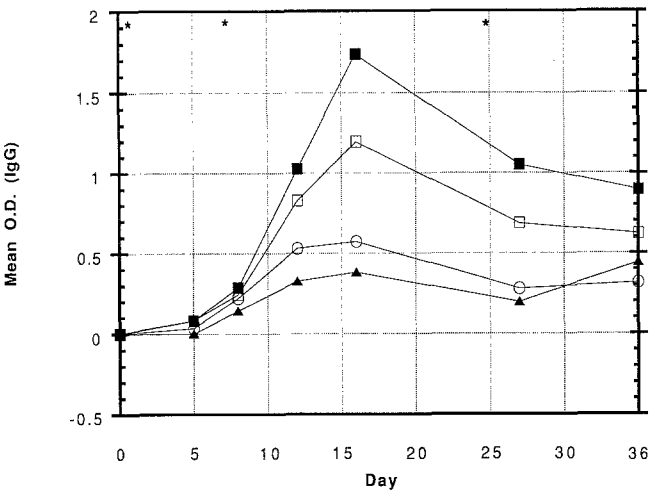

Fig. 7. Anti-keyhole limpet haemocyanin (KLH) IgG antibody response to recombinant human interleukin-2 administration. Marked increase in relative antibody levels compared to control dogs was observed at day 16 for dogs from groups 2 and 3 (6×10^5 and 6×10^6 IU/day, respectively; $p = 0.08$). O.D., optical density (absorption reading) recording of the spectrophotometric reader; *, day of administration of KLH; ▲, control group; o, group 1; □, group 2; ■ group 3.

Antigenic Specific IgG and IgM Responses. Anti-KLH IgG and IgM peak levels were dose related: that is, highest relative levels of antigen specific IgG and IgM were recorded in the highest dose group (group 3) followed by group 2, then group 1. Lowest peak levels were observed in the excipient control group (Figs. 6 and 7). Peak levels of anti-KLH IgG and IgM were markedly elevated from control values in groups 2 and 3 ($p = 0.08$), but not in group 1 ($p = 0.7$).

Discussion

Previous studies have demonstrated in vitro immunomodulating activity of rhIL-2 in the dog (Fenwick et al. 1988; Jardine et al. 1989; Mitchel et al. 1991; Raskin et al. 1991). The magnitude of the in vitro blastogenic or tumour cytotoxicity response of canine lymphocytes was dependent on the concentration of rhIL-2 (Fenwick et al. 1988; Raskin et al. 1991). In the present study, rhIL-2 induced in vivo immunomodulating effects in dogs. These effects were likewise most prominent in dogs that received relatively high doses of subcutaneous rhIL-2. In the present study, the most marked rhIL-2 augmented lectin blastogenic responses occurred in conA and PKW-stimulated lymphocytes from dogs administered the highest dose of rhIL-2 (group 3: 6×10^6 IU/kg; day 33). Enhanced B and T lymphocyte mitogenic activity in response to rhIL-2 stimulation has been reported in a wide variety of animal species (Fenwick et al. 1988). In addition, the present study, rhIL-2 induced increased antigen specific immunoglobulin secretion, thus effecting B lymphocyte stimulation in vivo. Other studies have demonstrated IL-2 response on B lymphocytes and IL2-induced immunoglobulin secretion (Ralph et al. 1984; Zubler et al. 1984).

Enhanced lymphocyte mitogenic stimulatory response and antigen-specific immunoglobulin production after rhIL-2 administration are indicative of potential application of this cytokine for treatment of immunosuppression in dogs. In addition, rhIL-2 may be useful for enhancement of the immune response to weak antigens. The immunomodulating activity of rhIL-2 in dogs may also be useful in an experimental setting, e.g. to stimulate immunological recovery after bone marrow transplantation. In human beings, B lymphocyte activity is frequently suppressed and there is weak immunoglobulin production after bone marrow transplantation (Lum et al. 1986; Lum 1987). The dog is frequently used as a preclinical model for development of bone marrow transplantation protocols; thus, administration of recombinant human IL-2 may be useful in the canine model to evaluate its effectiveness in enhancement of immune function after transplantation (Vriesendorp and van Bekkum 1980). Another important application in a canine preclinical marrow transplantation model would be assessment of the effects of rhIL-2 administration on development or possible augmentation of graft-versus-host disease, a severe complication of bone marrow transplantation mediated in part by donor T lymphocytes (Storb and Thomas 1985).

The most marked haematological effects of in vivo rhIL-2 administration were observed with absolute blood lymphocyte and eosinophil counts. In the highest dose group (group 3; 6×10^6 IU/kg), transient elevation of lymphoctye counts (350% of baseline) was observed 3 days after the last of five daily subcutaneous injections of rhIL-2. Lymphocyte counts returned to baseline levels within 2 weeks of the last rhIL-2 injection. This suggests that repeated series of rhIL-2 injections may be required to attain sustained elevated levels of circulating lymphocytes in dogs. A similar transient rebound phenomenon in circulating lymphocyte numbers has been observed in human beings after termination of rhIL-2 administration (Mier 1987). The increased number of lymphocytes had natural killer activity and enhanded LAK differentiation in vitro. Rebound lymphocytosis in the dog may allow for scheduling of leukopheresis soon after high-dose rhIL-2 administration to attain activated canine lymphocytes for experimental or therapeutic purposes.

The most remarkable alteration of circulating blood cells occurred in eosinophil counts. All dogs (9/9) administered rhIL-2 (6×10^4 to 6×10^6 IU/kg) developed transiently elevated absolute eosinophilia $>1 \times 10^3$ cells/μl. Peak eosinophil counts were recorded at the end of the five daily rhIL-2 injections and had resolved to baseline levels within 2 weeks. There was no apparent deleterious effect of the transient eosinophila in any of the dogs. Similar rises in eosinophil counts have been reported in dogs after administration of rhIL-2 by selective hepatic infusion and in cats (Da Pozzo et al. 1992; Tompkins et al. 1990).

In cats the increased eosinophil count was attributed to IL-2 induced hyperplasia of bone marrow eosinophil precursors. A similar response in humans after administration of rhIL-2 has been attributed to the release of eosinophil colony-releasing factor by IL-2 stimulated T lymphocytes (Lotze et al. 1986; Mier 1987).

Humans and mice develop a capillary leak syndrome in which extravasation of fluid into the interstitium of multiple organ systems occurs after IL-2 administration (Lotze et al. 1986; Rosenstein et al. 1986). Anasarca, interstitial pulmonary oedema, weight gain, and decreased serum albumin concentrations are common specific clinical complications in humans after IL-2 treatment. In the present study, the predominant adverse clinical response to rhIL-2 administration in dogs was diarrhoea; the severity of which was dose related, with resolution within 1 week after the last of five daily rhIL-2 injections. This may be a source of concern for future long-term rhIL-2 administration in dogs. Severe diarrhoea also occurs in human beings after rhIL-2 administration due possibly to the vascular effects of serotonin or histamine release from cytokine-stimulated basophils and mast cells (Subramanian and Bray 1987).

Lymphocyte counts were transiently decreased (within 8h) from the peripheral vasculature in all dose groups after rhIL-2 administration. In human beings, similar redistribution of lymphocytes from the peripheral vasculature occurs after rhIL-2 administration (Lotze et al. 1986). This was attributed to accumulation of activated cells within the liver and lungs as a result of loss of homing receptors for lymph nodes and increased binding activity of tissue endothelial cells for activated T lymphocytes (Rosenberg 1984; Gallatin et al. 1986; Mier 1987).

In summary, the present study demonstrates that rhIL-2 administration in dogs has in vivo immunomodulating effects. Many of these responses were similar to those observed in human beings after rhIL-2 administration. Transient clinical toxicity manifested predominantly by diarrhoea was observed in the dogs in a dose-limiting manner, however no alteration in serum biochemical profiles were observed when evaluated 24 days after the last rhIL-2 injection. Diarrhoea was most severe in dogs after five daily subcutaneous does of 6×10^6 IU/kg. Transient lymphocytosis, after initial redistribution of the peripheral circulating lymphocyte pool, and marked eosinophilia were observed in the rhIL-2 treated dogs. In addition, enhanced mitogen-induced lymphocyte blastogenesis and antigen-specific immunoglobulin production were the most impressive immunostimulatory effects recorded. Additional studies will demonstrate whether rhIL-2 may be useful in the dog as an experimental immunomodulating drug or applied clinically for treatment of specific immunodeficient states or neoplastic disease.

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