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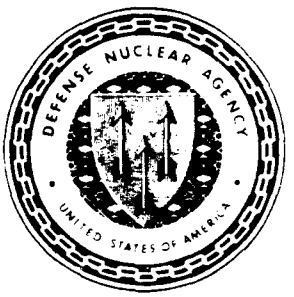

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Radiation-Induced Hemopoietic and Immune Dysfunction

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13. ABSTRACT *(Maximum 200 words)*

This work was aimed at investigating radiation-induced hemopoietic and immune dysfunction in the dog model. One project was to produce monoclonal antibodies directed against canine hemopoietic precursor cells and select for pluripotent stem cells. Antibodies obtained reacted with different myeloid and erythroid precursor cells; unfortunately, none of the antibodies were specific for pluripotent hemopoietic stem cells. Positive selections for these cells were performed using magnetic beads and an antibody against class II-antigen. Transplantation of class II positive marrow cells into otherwise lethally irradiated dogs led to sustained recovery in only 1 of 5 dogs.

A second project established and investigated long-term marrow cultures in the dog. Culture conditions were studied and optimized, and marrow cells were transplanted into otherwise lethally irradiated dogs to investigate stem cell survival in long-term cultures. Engraftment was observed only with short-term marrow cultures.

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PREFACE

Dogs are provided by the Center's canine breeding program and by breeding facilities licensed by the U.S. Department of Agriculture who have contracted to provide pedigreed dogs for research purposes. Dogs are housed in individual cages or runs according to the space recommendations of the "Guide for the Care and Use of Laboratory Animals." The Center's facility is accredited by the American Association for Accreditation of Laboratory Animal Care and is a U.S.D.A. registered research facility (#91-25). Animal facilities are cleaned by trained personnel on a daily basis. Animals are fed once daily and have continuous access to fresh, uncontaminated drinking water. All investigators are physicians or veterinarians and they are assisted by a chief animal technician who has been working in the canine research program for 25 years and has extensive experience in animal care and husbandry. All experimental dogs are seen twice daily by one of these individuals. Separate clinical charts are maintained on each dog. All animals that die unexpectedly or that are humanely euthanized undergo careful autopsy with subsequent review of histological specimens by trained pathologists. Carcasses are incinerated. Experimental protocols covering all the procedures performed are discussed at regular staff meetings and, after being approved by the principal investigator and senior staff members, are submitted to the Institutional Animal Care and Use Committee for approval. All research activities and procedures described above are conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council.

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Generation of monoclonal antibody 16-151-S3 followed the procedure initially developed by Koehler and Milstein (Nature 1975, 256: 495) and modified by Galfre and Milstein (Meth for Enzymol 1981, 73:1). Marrow cells were aspirated from both humeri of the irradiated dogs six days after TBI (total dose 9.2 Gy, dose rate 0.07 Gy per minute) without subsequent marrow transplant. For marrow aspiration, dogs were anesthetized by intravenous injection of sodium pentobarbital at 24 mg/kg. The aspiration sites on both humeri of the dogs were shaved and aseptically cleansed with a povidone-iodine scrub and an alcohol rinse. Marrow cells obtained six days after TBI were used as antigen for the immunization of mice. At this time after TBI, most hemopoietic cells are destroyed except for a small number of radiation-resistant cells, some of which are thought to play a role in marrow graft rejection. Originally, we planned to use this antigen for fusion in order to obtain monoclonal antibodies against cells mediating graft rejection. One of the clones derived from this fusion, 16-151-S3, produced monoclonal antibodies which reacted strongly (98%) with granulocytes as shown by immunofluorescence analysis with the cell sorter FACS-440. Monoclonal antibodies of clone 16-151-S3 also bound strongly to marrow cells (54%) and monocytes (59%) but not to lymph node lymphocytes (0%). Determination of antibody class by immunodiffusion (Ouchterlony) technique showed this antibody to be IgG_{2B}. To further determine which type of marrow cells monoclonal antibody 16-151-S3 was reacting with, we sorted marrow cells into monoclonal antibody positive and negative cells using the FACS-440 cell sorter. Cytospins of the positively sorted marrow cells showed 87% neutrophils, 7% myelocytes, 2% promyelocytes, 1% myeloblasts, 1% pronormoblasts, 2% normoblasts, and no lymphocytes, whereas cytospins of the antibody-negative marrow cells contained 18% lymphocytes, 4% normoblasts, and 78% erythrocytes. When positively and negatively sorted marrow cells were cultured in CFU-GM assay, only monoclonal antibody positive marrow cells showed growth of CFU-GM colonies.

We then investigated whether monoclonal antibody 16-151-S3 reacts with pluripotent hematopoietic stem cells. This was done by treating marrow cells in vitro with monoclonal antibody and rabbit complement before infusion into lethally irradiated dogs (9.2 Gy total dose, dose rate 0.07 Gy per minute). We studied whether in vitro treatment interfered with the ability of autologous marrow to protect dogs against radiation-induced marrow aplasia. Two dogs

fractions using the FACS-440. Unsorted, monoclonal antibody-positive and monoclonal antibody-negative marrow cells were then assayed for CFU-GM colony growth. DM5-positive marrow cells did not produce CFU-GM colonies, whereas DM6 and DM10-positive marrow cells produced CFU-GM colonies. DM7- and DM8-positive marrow cells as well did not produce any CFU-GM colonies.

We then investigated whether one of these antibodies, DM5, recognizes canine pluripotent stem cells. This was done by treating marrow cells in vitro with DM5 antibody and rabbit complement before infusion into lethally irradiated (920 cGy) marrow donors. Two dogs were entered into this experiment and received 6 and 8 x 10^7 marrow cells, respectively, treated with DM5 and complement. In both experiments no DM5-positive marrow cells could be detected by FACS analysis after treatment with antibody and complement. Recovery of white blood cells and platelet counts was comparable to the recovery in control dogs receiving untreated autologous marrow transplants. We concluded from these experiments that DM5 seems not to react with canine pluripotent stem cells. As DM5 binds to 33% of marrow cells, but not to pluripotent stem cells, it may be useful to pre-enrich for stem cells by treating marrow with antibody and complement.

1.3 RADIOIMMUNE PRECIPITATION OF DM5 ANTIGENS.

After SDS-PAGE under nonreducing conditions, three bands were seen in the DM5 immunoprecipitate of marrow cells with apparent molecular weights of 19, 21 and 23 kD. Under reducing conditions, an additional small molecular weight band was observed that migrated beyond the 14.3 kD molecular weight. Occasionally a faint band of 16-17 kD was seen under reducing conditions. To address the possibility that the small molecular weight component represented a disulfide-linked polypeptide which associated, in a nonspecific manner, with gp $19,21,23^{DM5}$ at the time of cell lysis, cells were labeled and incubated for 10 minutes in 10 mM Iodacetamide (Sigma) in PBS prior to detergent solubilization. Results identical to those obtained under reducing conditions were obtained when this alkylating agent was used to prevent artifactual disulfide associations that can occur during cell solubilization. When DM5 RIPs were analyzed by 2-dimensional PAGE under reducing conditions, gp $19,21,23^{DM5}$ all migrated at a pI of 5.9, and a small molecular weight fragment had a pI of 6.2. This observation suggests that the three larger components remained associated during detergent solubilization of marrow cells and during the first isolectric focusing dimension of 2D-PAGE as well, but were separated only by SDS denaturation. Alternatively, the three freely soluble components may possess a high degree of structural homology, but differ in the relative contribution to apparent molecular mass of uncharged substituents, such as neutral oligosaccharides. To determine if the three molecules (19, 21, and 23 kD) were glycosylation variants of each other, N-Glycanase digestion was performed on immunoaffinity isolated molecules. N-Glycanase completely removes N-linked oligosaccharides, including high mannose chains from glycoproteins. After overnight digestion, all three species resolved to a single 15 kD band This result suggests that

SECTION 2

POSITIVE SELECTION FOR CANINE MARROW STEM CELLS USING SEPARATION WITH MAGNETIC BEADS

As described previously (Blood 69:165-172, 1987), we have shown that canine pluripotent hemopoietic stem cells express a class II antigen as recognized by the antibody 7.2. We have demonstrated that 7.2-positive marrow cells, separated with FACS, can rescue otherwise lethally irradiated marrow donors. As the positive sort of a marrow transplant with FACS is very time-consuming and expensive, we were interested to find out whether we can use the magnetic bead technique for positively sorting marrow transplants. We first established in vitro the positive separation method using magnetic beads coated with sheep antimouse antibody (Dyna beads M-450) and a neodymium-iron-boron magnet (Magnet Development Ltd, Swindon, Wales, England). We used the anti-class II antibody 7.2 for positive bone marrow separation, which we have shown to be expressed on canine pluripotent hemopoietic stem cells. All separation steps were done on ice or at 4°C. Bone marrow cells were first separated on a Ficoll-Hypaque gradient, washed, and counted. They were then incubated with the anti-class II antibody 7.2 for 20 minutes, washed twice with RPMI containing 10 percent fetal calf serum. To test how many cells were viable and how many viable cells were rosette-forming, i.e., binding three or more beads per cell, approximately 25,000 to 50,000 cells were pipetted into a well of a round-bottom microplate. Excess number of beads (10-20 times the number of cells) were added. The cell bead mixture was spun down in a cold centrifuge for one minute at 1200 rpm, and afterwards resuspended by adding one or two droplets of ethidium bromide acridine orange solution to the cell bead mixture. The suspension was then placed in a Burker hemocytometer and counted in a fluorescence microscope to determine the relative number of rosette-forming cells. Rosette-forming cells were defined as cells which have bound three or more beads. Live cells stained green and dead cells stained red. Approximately 1000 cells were counted to obtain a fairly accurate relative number of rosette-forming cells. After the relative number of rosette-forming cells had been established, the amount of beads needed was calculated according to the formula: number of total mononuclear cells x percent of rosette-forming cells equals the number of target cells. For example, if a ratio of beads to target cells of 3:1 was wanted, beads at the amount of 3 times the number of target cells needed to be added to the marrow cells, which were set up at a concentration of 40,000,000 - 60,000,000 per ml. After having added the beads to the cell suspension, the bead/cell mixture was centrifuged for one minute at 1200 rpm. After centrifugation, the volume was increased by adding 3-5 cc of RPMI containing 10% fetal calf serum and DNAse. The cell suspension was carefully mixed and the tube containing the cell/bead mixture was then laid sideways on the magnet. After two to three minutes, the tube and the magnet were turned around so that the magnet was now on top of the tube. The unattached supernatant containing the negative population was aspirated carefully. The remaining positive cell population was then resuspended gently and the number of cells binding three or more beads counted. This separation step with the magnet was

The numbers of 7.2-positive nucleated marrow cells infused into the three dogs after TBI were between 0.03 and 0.06 x 10^8 mononuclear cells/kg body weight. These numbers were 5-10 times less than the one given to the dog who showed sustained and complete hemopoietic recovery. The low cell number transplanted may explain why we did not see any signs of hemopoietic recovery in these last three dogs. The low number of cells transplanted was due to the low amount of cells obtained at the time of marrow aspiration and to the low number of cells recovered after cell separation with the magnetic beads. It therefore seems necessary to further improve the number of cells originally aspirated, as well as the number of cells recovered after cell separation with the magnetic beads.

Table 1. Comparison of canine long-term marrow cultures established at identical culture conditions.

Week	Flask 1		Flask 2		Flask 3		Flask 4		Flask 5	
	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM
1	200	0	152	0	163	0	152	0	220	850
2	ND		ND		ND		ND		ND	
3	100	0	80	208	80	416	40	1612	80	2600
4	45	1112	45	3920	75	3218	40	2704	60	3354
5	30	1989	30	2184	60	1560	10	247	45	ND
6	40	5304	40	1248	110	3003	52	1960	50	2080
7	80	2338	50	1755	100	1300	120	2808	65	592
8	37	1539	110	1001	130	1352	55	429	62	887
Σ	532	12282	507	10316	718	10849	469	9760	582	10363

All cultures were recharged one week after establishing an adherent cell layer with 4.5×10^7 MNC containing 585 CFU-GM.

ND = Not done

Σ = Sum of weekly counts

Table 3. Comparison of different culture media in canine long-term marrow culture.

Week	RPMI Medium 1640		McCoy's 5A Medium		Alpha Medium		Fischer's Medium	
	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM
1	358	1,862	340	9,724	406	6,334	291	1,892
2	350	19,565	20	1,014	32	998	17	309
3	10	221	12	412	18	374	5	143
4	32	541	14	109	5	5,230	11	143
5	ND		ND		ND		ND	
6	22	114	16	125	16	0	13	0
Σ	772	22,303	402	11,384	477	12,936	337	2,487

All cultures were recharged one week after establishing an adherent cell layer with 6.4×10^7 MNC containing 41,600 CFU-GM.

ND = Not done

Σ = Sum of weekly counts

Table 4. Comparison of different amounts of hydrocortisone in canine long-term marrow culture.

Week	No Hydrocortisone		0.05 μM Hydrocortisone		0.1 μM Hydrocortisone		0.2 μM Hydrocortisone		0.4 μM Hydrocortisone	
	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM	Nonadherent cells ($\times 10^5$)	CFU-GM
1	1,090	670,241	890	259,168	1,000	44,200	1,160	1,508	1,130	30,849
2	130	14,027	60	53,508	96	12,979	62	37,398	50	2,990
3	36	24,617	15	49,937	10	30,914	9	25,459	3	0
4	15	15,113	24	38,314	18	30,046	8	15,215	6	3,726
5	22	22,108	24	19,375	35	30,940	6	5,561	2	1,250
Σ	1,293	746,106	1,013	417,302	1,159	149,079	1,245	85,141	1,191	38,815

All cultures were recharged one week after establishing an adherent cell layer with 4.5×10^7 MNC containing 257,400 CFU-GM.

Σ = Sum of weekly counts

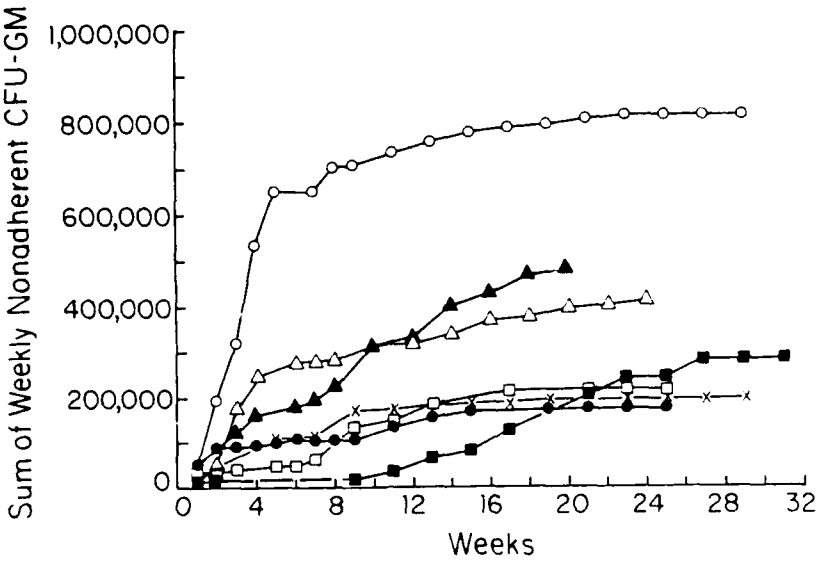

Figure 1. Cumulative weekly CFU-GM in the nonadherent cell fraction of canine long-term marrow cultures recharged with autologous or allogeneic DLA-nonidentical marrow cells after 1, 2, or 3 weeks. Cultures were started by incubating 6 x 10^7 MNC from the same marrow aspiration in 75 cm^2 canted-neck flasks at 2 x 10^6 MNC/ml in RPMI-1640 medium supplemented with 20% prescreened heat-inactivated horse serum. Flasks were either not recharged (*) or boosted after 1, 2, or 3 weeks with 2 x 10^7 MNC derived either from the same donor as used for establishing the stromal cell layer (○, 1-week boost; △, 2-week boost; and □, 3-week boost) or from a DLA-nonidentical unrelated donor (●, 1-week boost; ▲, 2-week boost; and ■, 3-week boost). At weekly intervals nonadherent cells were plated in triplicate in CFU-GM assay. Each point represents the cumulative number of weekly CFU-GM.

need to be autologous compared to the cells of the adherent layer or whether they can be allogeneic DLA-nonidentical. Cultures were started by incubating 6 x 10^7 MNC from the same marrow aspiration in culture flasks. Flasks were either not recharged or boosted after 1, 2, or 3 weeks with 2 x 10^7 MNC derived either from the same donor used for establishing the stromal cell layer or from a DLA-nonidentical unrelated donor. Results are shown in Figure 1. When cultures were recharged one week after establishing the stromal layer, the flask recharged with autologous marrow showed a higher sum of weekly CFU-GM counts in the nonadherent cell fraction compared to the flask boosted with DLA-nonidentical marrow (at week 20, the week before the first flask became contaminated, 796,337

long-term marrow culture seem to lose their ability to protect dogs against irradiation induced marrow aplasia. This finding was in contrast to our previous finding that we could grow CFU-GM colonies from long-term cultures for up to 31 weeks. Most probably pluripotent hemopoietic stem cells seem to lose their ability for self-renewal rather quickly under the present long-term culture conditions by developing into committed precursor cells. On the other side, these precursor cells, though committed, were able to produce CFU-GM for up to eight months.

To further clarify the contrasting results of our in vitro and in vivo studies, we then addressed the question whether failure of engraftment of autologous marrow cells cultured for more than six days might be due to the lack of accessory cells which may not survive in long-term marrow cultures. For instance, it has been described that lymphocytes are rapidly lost in long-term marrow cultures (Mauch et al., Blood 63:112, 1984). We therefore used autologous lymph node lymphocytes as possible source of accessory cells. Marrow cells were cultured in long-term culture for eight days, harvested and infused into the lethally irradiated marrow donor together with autologous lymph node lymphocytes. The number of cultured mononuclear marrow cells infused was 17 x 10^6 per kg body weight and the number of lymph node lymphocytes was 6 x 10^6 per kg body weight. The dog never showed any signs of engraftment and died on day 13 after transplantation due to sepsis. A second dog received as well marrow cells cultured for eight days in long-term marrow culture, but in this experiment stem cell-free peripheral blood lymphocytes were used as possible source of accessory cells assuming that this would be more physiological. To obtain peripheral blood lymphocytes depleted of hemopoietic stem cells, we first separated blood cells by density gradient centrifugation over Ficoll-Hypaque. Interface cells were then treated with hemolytic buffer (0.155 M per l of ammonium chloride) and separated by a discontinuous albumin density gradient as described by Dicke (Transplantation 6:562, 1968). The peripheral blood leukocytes from fraction five of albumin density gradient are depleted of hemopoietic stem cells insofar as they failed to achieve hemopoietic reconstitution after lethally irradiation (Schuening et al., Blood 69:165, 1987). Marrow cells were cultured in long-term cultures for eight days, harvested, and then infused into the lethally irradiated marrow donor together with autologous peripheral blood lymphocytes from fraction five of the albumin density gradient. The number of cultured mononuclear marrow cells infused was 76 x 10^6 per kg body weight and the number of PBL from fraction five was 9 x 10^6 per kg body weight. The marrow cell number transplanted always results in sustained engraftment when using fresh autologous marrow cells. However, similar to the previous experiment where we gave autologous lymph node cells in addition to marrow cells cultured for eight days, this dog never showed any signs of engraftment as well and died on day 12 due to marrow aplasia. This result confirmed our previous observation and showed that failure of engraftment using marrow cells cultured for more than six days in long-term marrow culture is probably due to insufficient numbers of pluripotent hemopoietic stem cells rather than to lack of accessory cells.

In summary, using the current culture conditions, we obtained sustained engraftment of marrow cells cultured in long-term marrow culture in one of one dog receiving marrow cells cultured for four days and in three of five dogs transplanted with autologous marrow which had been cultured for six days in long-term marrow culture. These results are inferior to those obtained in mouse and man. In mice, sustained marrow constitution has been seen after transplantation of marrow cultured in long-term marrow culture for three weeks (Transplantation 35:624, 1983). Patients receiving marrow which has been kept in long-term culture for ten days have shown successful engraftment as well (Lancet 1:294, 1986). Our current results in dogs need to be improved. We therefore started to investigate whether the addition of hemopoietic growth factors to long-term marrow cultures improve the transplantation results.

3.9 EFFECT OF RECOMBINANT HEMOPOIETIC GROWTH FACTORS ON CANINE LONG-TERM MARROW CULTURES.

The effect of recombinant hemopoietic growth factors on canine long-term marrow cultures was tested using the following experimental setting. Identical 75 cm^2 tissue cultures flasks were inoculated with 60 x 10^6 bone marrow mononuclear cells in 30cc media and boosted with 60 x 106 marrow buffy coat mononuclear cells one week later. Fifty percent of the culture media was replaced weekly and growth factors of interest were also added at weekly intervals. A CFU-GM assay was performed weekly on nonadherent cells. In repeated long-term cultures with marrow obtained from different normal beagles, we observed a stimulatory effect with recombinant human IL-3 on CFU-GM production in a dose-dependent fashion without evidence of stem cell exhaustion over an 8-week interval (Figure 2). This is consistent with the hypothesis that IL-3 affects a multipotential hemopoietic precursor cell capable of at least limited self-renewal.

The effect of recombinant human G-CSF has been variable. At 10^{-10} gram per ml, we have inconsistently observed a stimulatory effect on CFU-GM production. However, at doses of 10^{-8} and 10^{-6} gram per ml, a significant negative effect on CFU-GM production was clearly evident (Figure 3). This suggests that G-CSF at high doses causes differentiation of committed progenitor cells to beyond the CFU-GM stage. At low doses, some effect on early progenitors toward self-renewal, or limited differentiation is probable.

The effect of recombinant human GM-CSF has paralleled that observed with recombinant human G-CSF. At 10^{-10} gram per ml, marked stimulation was seen in CFU-GM production (Figure 4). However, doses of 10^{-8} and 10^{-6} gram per ml induced marked inhibition of colony production. Results obtained with recombinant canine G-CSF were similar to the results obtained with recombinant human G-CSF. We found an approximately 60% cumulative increase in CFU-GM production over 8 weeks in flasks recharged weekly with recombinant canine G-CSF at doses of 10^{-12} and 10^{-10} g/ml compared to controls (Figure 5). However, there was a markedly reduced cumulative CFU-GM production in the flasks receiving the higher doses of recombinant canine G-CSF

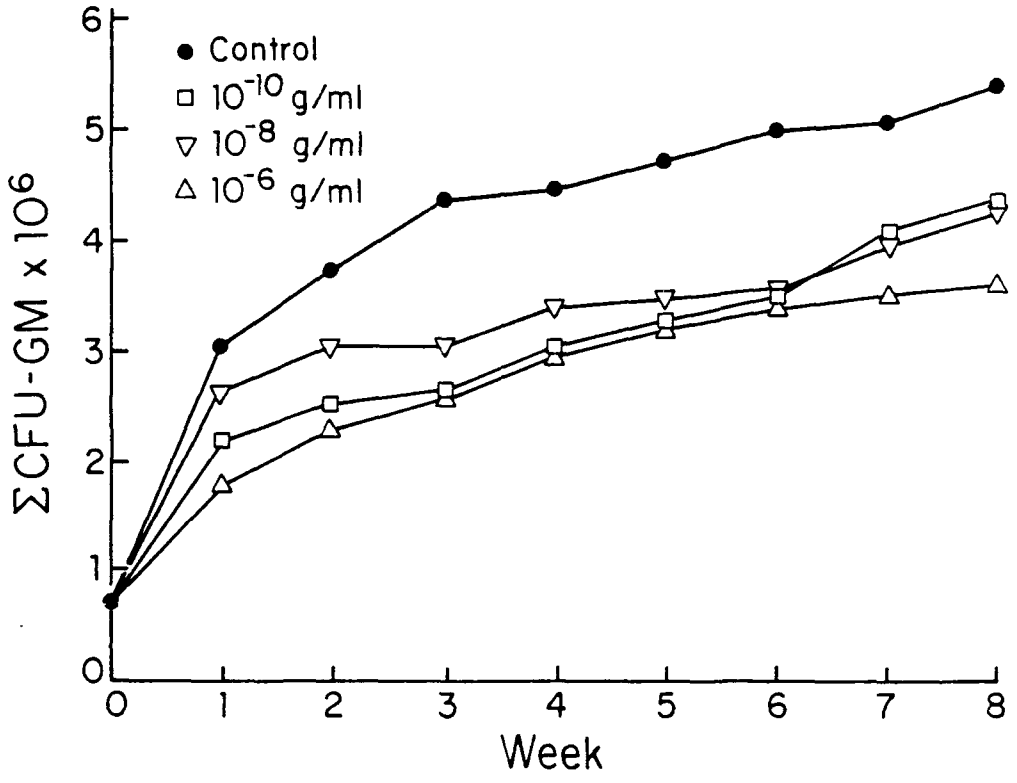

Figure 3. Cumulative weekly CFU-GM in the nonadherent cell fraction of canine long-term marrow cultures recharged with autologous marrow cells after 1 week and fed weekly with recombinant human G-CSF at 10^{-6} g/ml (△-△), 10^{-8} g/ml (▽-▽), 10^{-10} g/ml (□-□). Control was fed with medium only (●-●).

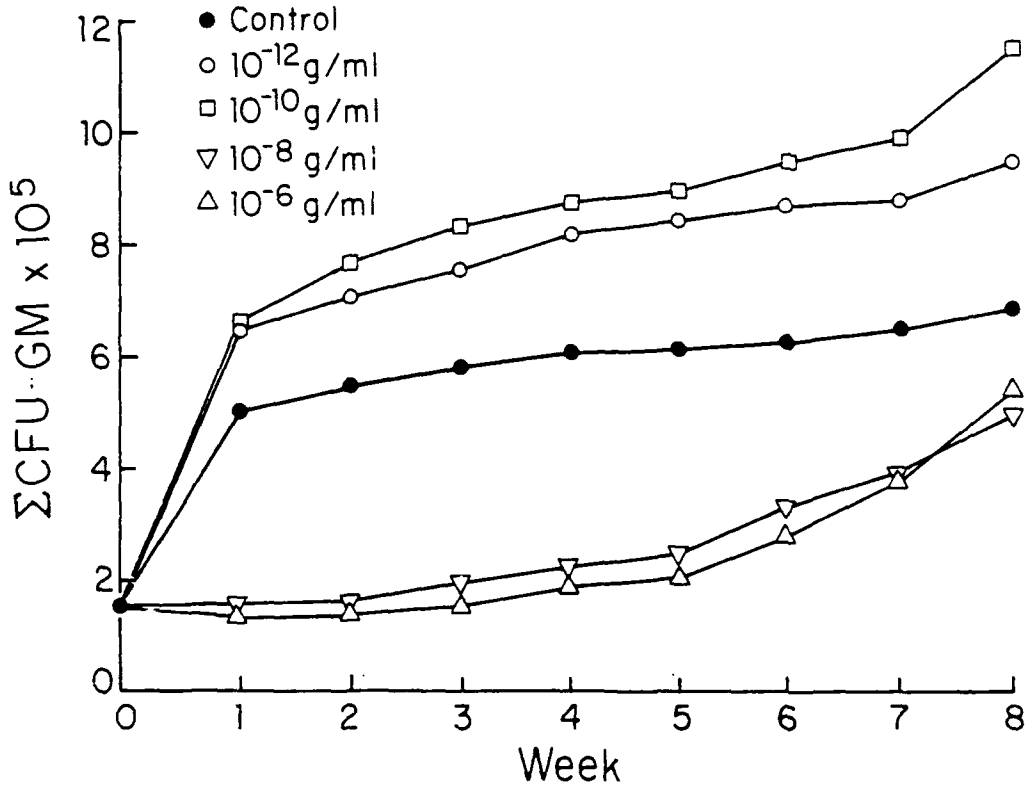

Figure 5. Cumulative weekly CFU-GM in the nonadherent cell fraction of canine long-term marrow cultures recharged with autologous marrow cells after 1 week and fed weekly with recombinant canine G-CSF at 10^{-6} g/ml (△-△), 10^{-8} g/ml (▽-▽), 10^{-10} g/ml (□-□). Control was fed with medium only (●-●), and 10^{-12} g/ml (○-○).

SECTION 4

STIMULATION OF CANINE HEMATOPOIESIS BY RECOMBINANT HUMAN IL-3

4.1 STIMULATION OF CANINE CFU-GM COLONY FORMATION BY RECOMBINANT HUMAN IL-3.

Recombinant human IL-3 (rhIL-3) was added to CFU-GM cultures at different concentrations. Culture plates without any addition of growth factor served as negative controls and plates with 10% postendotoxin dog serum as positive controls. In five separate experiments, an up to 29-fold stimulation of canine CFU-GM colony formation by rhIL-3 was observed compared to the negative controls (Figure 6). In a separate experiment, rhIL-3 was either incubated for 30 minutes with anti-rhIL-3 antiserum (diluted 1:200) or heat-inactivated at 100°C for 30 minutes before adding the growth factor to the culture plates. The unmanipulated rhIL-3 stimulated CFU-GM colony formation up to seven-fold compared to the negative controls. rhIL-3 treated with either anti-rhIL-3 antiserum or heat-inactivation did not show any stimulatory activity compared to the colony growth of the negative control (Table 6). Anti-rhIL-3 antiserum by itself, did not have any effect on CFU-GM colony growth. These results are consistent with the notion that rhIL-3 specifically interacts with canine CFU-GM.

4.2 STIMULATION OF HEMATOPOIESIS IN DOGS BY CONTINUOUS INTRAVENOUS INFUSION OF RhIL-3.

Five dogs were treated with 30, 60 (two dogs) 120 and 240 μg rhIL-3 per kg/day given as continuous intravenous infusion for 14 days. rhIL-3 was well tolerated and no systemic toxicity was observed over the dose range studied. No stimulation of hematopoiesis was seen at the dose of 30 μg/kg per day (Figure 7). At 60, 120, and 240 μg/kg per day, neutrophil counts reached levels by day 14 which were two times higher than the counts of two control dogs receiving either 60 μg heat-inactivated rhIL-3 per kg/day for 14 days (range indicated by two horizontal lines) or 10 μg human serum albumin per kg/day. After discontinuation of rhIL-3, neutrophil counts remained increased for 10 to 14 days before returning to the counts of the control dogs. Peripheral monocytes were increased up to three-fold. No significant changes were observed in eosinophil, lymphocyte, reticulocyte or hematocrit levels. The effect of rhIL-3 on platelets is shown in Figure 8. At 30 and 60 μg rhIL-3 per kg/day, the platelet counts did not change compared to the control dog receiving 60 μg heat-inactivated rhIL-3 per kg/day (range indicated by two horizontal lines). The dog receiving 120 μg/kg per day showed a slight increase of platelets above the upper limit of the control seven days after the discontinuation of rhIL-3. At a dose of 240 μg rhIL-3 per kg/day, platelet counts were increased two-fold above the control five days after stopping the rhIL-3.

Marrow biopsies were taken before and after 14 days of rhIL-3 infusion and slides were evaluated without knowledge of treatment

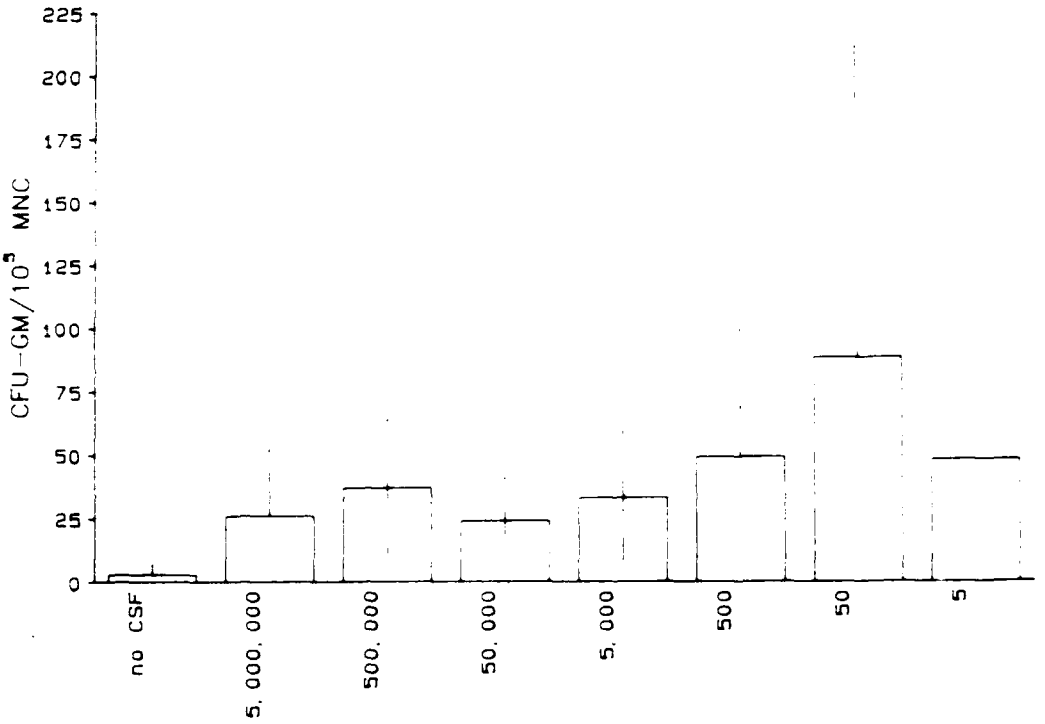

Figure 6. Effect of recombinant human IL-3 (rhIL-E) on canine CFU-GM colony formation. 3×10^4 mononuclear marrow cells (MNC) depleted of monocytes and T-lymphocytes have been cultured per plate for 10 days in agar medium without and with addition of rhIL-3 at decreasing concentrations. Cultures were plated in triplicate. Data represent mean value ± standard deviation of five separate experiments.

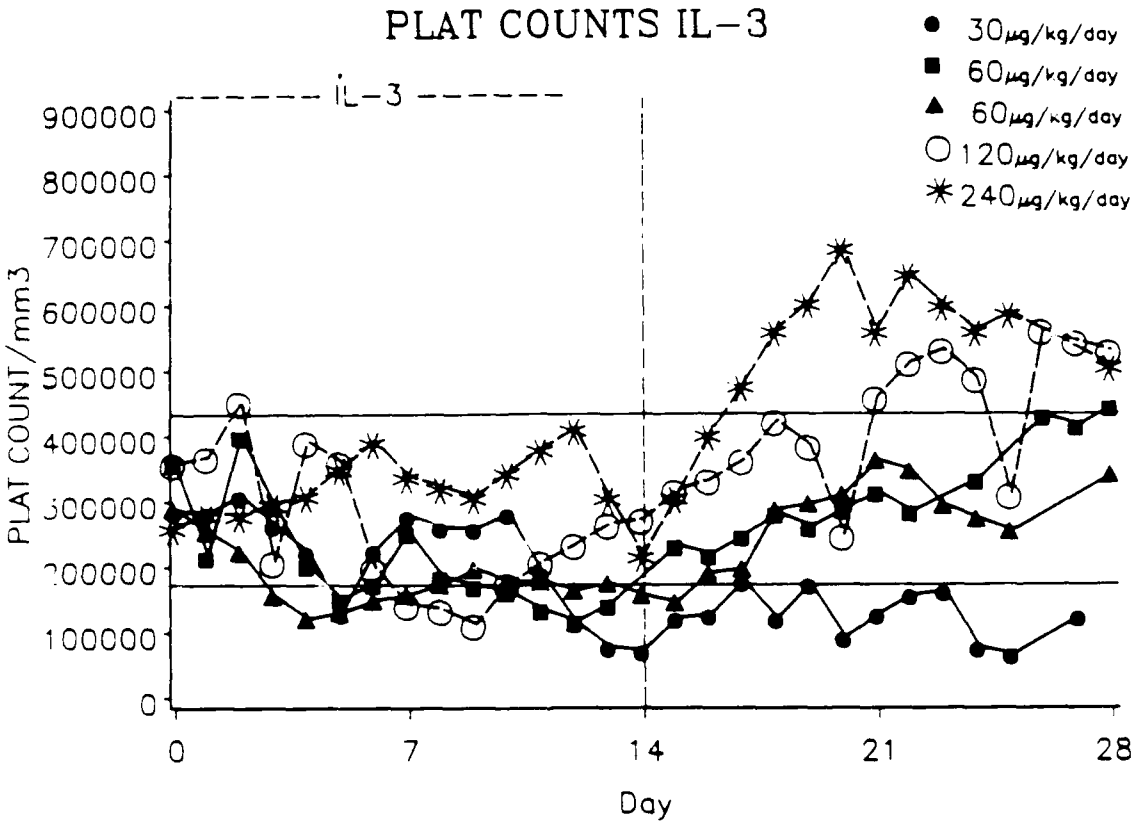

Figure 8. Effect of continuous (14-day) intravenous infusion of rhIL-3 given at 30, 60, 190 or 240 µg/kg/day on the peripheral blood platelets of five normal dogs. The two horizontal lines indicate the range of peripheral blood counts of a control dog given 60 µg heat-inactivated (100°C 30') rhIL-3 per kg/day for 14 days.

SECTION 5

STIMULATION OF CANINE HEMATOPOIESIS BY RECOMBINANT GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR

5.1 STIMULATION OF CANINE CFU-GM COLONY FORMATION BY RECOMBINANT HUMAN GM-CSF.

Recombinant human GM-CSF (rhGM-CSF) was added to the CFU-GM cultures at different concentrations. Culture plates without any addition of growth factor served as negative control and plates with 10% postendotoxin dog serum as positive control. In two separate experiments, an up to tenfold stimulation of canine CFU-GM colony formation by rhGM-CSF was observed compared to the negative control (Figure 9). In a separate experiment, rhGM-CSF was either incubated for 30 minutes with anti-rhGM-CSF antiserum (diluted 1:100) or heat-inactivated at 100°C for 30 minutes before adding the growth factor to the culture plates. The unmanipulated rhGM-CSF stimulated CFU-GM colony formation up to fourfold compared to the negative control. rhGM-CSF treated with either anti-rhGM-CSF antiserum or heat-inactivation did not show any stimulatory activity above the colony growth in the negative control (Table 8). Anti-rhGM-CSF antiserum by itself did not have any effect on CFU-GM colony growth. The results are consistent with the notion that rhGM-CSF specifically interacts with canine CFU-GM.

5.2 STIMULATION OF HEMATOPOIESIS IN DOGS BY CONTINUOUS IV INFUSION OF RhGM-CSF.

Three dogs were treated with 10, 30, or 90 μg rhGM-CSF per kg/day by continuous IV infusion for 14 days. RhGM-CSF were tolerated and no systemic toxicity was observed over the dose range studied. Dogs showed increased neutrophil counts within 24 hours of starting the infusion (Figure 10a). By day 14, neutrophil counts reached levels that were three to six times higher than the preinfusion counts and those of two control dogs receiving either continous IV infusion of 10 μg human serum albumin per kg/day for 14 days (range indicated by two horizontal lines) or 30 μg heat-inactivated rhGM-CSF per kg/day. The marrows in rhGM-CSF-treated dogs on day 14 were hypercellular with myeloid hyperplasia and left-shifted granulocytopoiesis. After discontinuation of rhGM-CSF, the neutrophil counts returned to control levels within 3 to 7 days. Whereas neutrophil counts increased at all three dosages of rhGM-CSF to about the same extent, monocyte and lymphocyte counts were markedly increased (eight and two- to fourfold, respectively) only at the two higher rhGM-CSF dosages (Figures 10b and c). Eosinophil and reticulocyte counts and hematocrits during rhGM-CSF infusion were similar to the preinfusion levels and the levels in the control dogs receiving human serum albumin or heat-inactivated rhGM-CSF.

The three dogs receiving active rhGM-CSF showed declines in platelet counts with nadirs ranging from 5,000 to 15,000/mm^3 (Figure 10d). After discontinuing the infusion of rhGM-CSF, platelet counts

Table 8. Stimulation of canine CFU-GM colony formation by rh GM-CSF.

	CFU-GM/10^5 MNC								
	No CSF	$5x10^{-6}$ g/ml GM-CSF	$5x10^{-7}$ g/ml GM-CSF	$5x10^{-8}$ g/ml GM-CSF	$5x10^{-9}$ g/ml GM-CSF	$5x10^{-10}$ g/ml GM-CSF	$5x10^{-11}$ g/ml GM-CSF	$5x10^{-12}$ g/ml GM-CSF	10% Peds
GM-CSF	19±4	23±3	34±1	34±3	69±7	81±10	68±17	40±3	372±76
Rabbit αGM-CSF + GM-CSF	19±4	11±3	20±6	15±6	11±6	5±3	6±0	13±4	—
Heat-inactivated GM-CSF (100°C, 30')	19±4	3±1	13±4	11±3	17±8	8±4	5±3	6±4	—

3×10^4 mononuclear marrow cells depleted of monocytes and T-lymphocytes have been cultured per plate for 10 days in agar medium without and with rh GM-CSF at decreasing concentrations. Cultures were plated in triplicate. Colony numbers represent mean value ± 1 standard deviation.

Peds = Postendotoxin dog serum
αGM-CSF = anti GM-CSF antiserum

increased rapidly above preinfusion levels. By comparison, the platelet counts of a saline control remained virtually unchanged, whereas the counts of a human serum albumin control decreased after 10 days of infusion to about 100,000/mm^3. The control dog receiving heat-inactivated rhGM-CSF also had a decrease of platelets to about 100,000/mm^3, but this decrease occurred 4 days after rhGM-CSF infusion. Bone marrow histology on day 14 of rhGM-CSF infusion showed normal number and morphology of megakaryocytes. Preliminary results of platelet survival studies using ^{51}Cr-labeled autologous platelets suggest that platelet survival is decreased to 50% of normal in the first week after starting rhGM-CSF infusion compared to normal survival times in dogs given saline or human serum albumin.

5.3 STIMULATION OF HEMATOPOIESIS IN DOGS BY INTERMITTENT SUBCUTANEOUS INJECTION OF RhGM-CSF.

One dog was treated with 30 μg rhGM-CSF per kg/day and two dogs received 180 μg rhGM-CSF per kg/day by intermittent subcutaneous injections three times a day for 14 days. The subcutaneous administration of rhGM-CSF was well-tolerated and no systemic toxicity was observed over the dose range studied. rhGM-CSF at the dose of 30 μg/kg per day SQ increased the neutrophil counts twofold compared to the preinfusion counts and those of a control dog receiving 10 μg human serum albumin per kg/day by continuous IV infusion for 14 days (range indicated by two horizontal lines) (Figure 11a). Monocyte and lymphocyte counts were not increased (Figure 11b and c). A SQ dose of 180 μg rhGM-CSF per kg/day increased the number of neutrophils fivefold above control (Figure 11a), and monocyte and lymphocyte counts were increased fivefold and fourfold, respectively (Figure 11b and c). Compared to results obtained by continous IV infusion of 30 μg rhGM-CSF per kg/day, the intermittent SQ injection of the same amount of rhGM-CSF seemed to be less effective in stimulating peripheral blood counts (Figure 11a-c). Platelet counts decrease to only about 100,000/mm^3 when 30 μg/kg per day of rhGM-CSF was given SQ (Figure 11d). With a dose of 180 μg/kg per day, platelets declined to 10,000/mm^3 similar to what was seen with continuous IV infusion of 30 μg/kg per day.

SECTION 6

STIMULATION OF CANINE HEMATOPOIESIS BY RECOMBINANT HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR

6.1 EFFECTS OF RECOMBINANT HUMAN G-CSF IN NORMAL DOGS.

Two dogs were treated with either 10 or 100 μg recombinant human G-CSF (rhG-CSF)/kg per day subcutaneously twice a day for 14 days. RhG-CSF was well-tolerated and no systemic toxicity was observed. Both dogs showed increased peripheral blood neutrophil counts within 24 hours of starting the injections of the growth factor (Figure 12a). By day 14, neutrophils reached levels that were eight to ten times higher than the preinfusion counts and those of two control dogs receiving either 60 μg heat-inactivated rhIL-3 per kg/day for 14 days (range indicated by two horizontal lines) or 10 μg human serum albumin per kg/day. After discontinuation of rhG-CSF the neutrophil counts returned to control levels within three days. Peripheral blood monocytes were increased four- to sixfold (Figure 12b). Lymphocyte counts remained unchanged at 10 μg rhG-CSF per kg/day and increased threefold at 100 μg rhG-CSF per kg/day (Figure 12c). No significant changes were observed in platelet counts (Figure 12d), eosinophil, reticulocyte or hematocrit levels. Marrow biopsies were taken before and after 14 days of rhG-CSF injections, and slides were evaluated without knowledge of treatment modality. Compared to the normocellular marrow histology before rhG-CSF treatment, the marrows of the rhG-CSF-treated dogs were hypercellular with myeloid hyperplasia and left-shifted granulopoiesis.

6.2 EFFECTS OF RhG-CSF IN DOGS AFTER LETHAL TBI.

In studies funded by a separate grant, we have investigated the survival of dogs given TBI, no marrow infusion and optimal supportive care. We found that recovery of dogs is uneventful after 200 cGy of TBI administered at a rate of 7 or 10 cGy per minute. Approximately 75% of dogs die of marrow aplasia and peripheral pancytopenia after 300 cGy of TBI. All dogs died following 400 cGy or higher doses of TBI. All dogs survived when given autologous marrow transplantation following TBI. We also found that dogs given 450 cGy TBI and DLA-identical marrow transplants showed transient allogeneic engraftment followed by rejection of the graft. One of these dogs was killed with IV sodium pentobarbital for purposes of obtaining tissue samples, one died with intermittent infection, and three survived with autologous marrow recovery as documented by blood genetic markers. We saw a similar phenomenon even after 600 cGy TBI. While 4 out of 7 dogs receiving this dose of TBI and DLA-identical marrow transplants showed sustained engraftment, one of the three dogs rejecting the marrow graft survived with autologous recovery. These findings show that endogenous hematologic recovery is possible even after an otherwise lethal dose of TBI of up to 600 cGy if dogs are given support through a transient DLA-identical marrow graft.

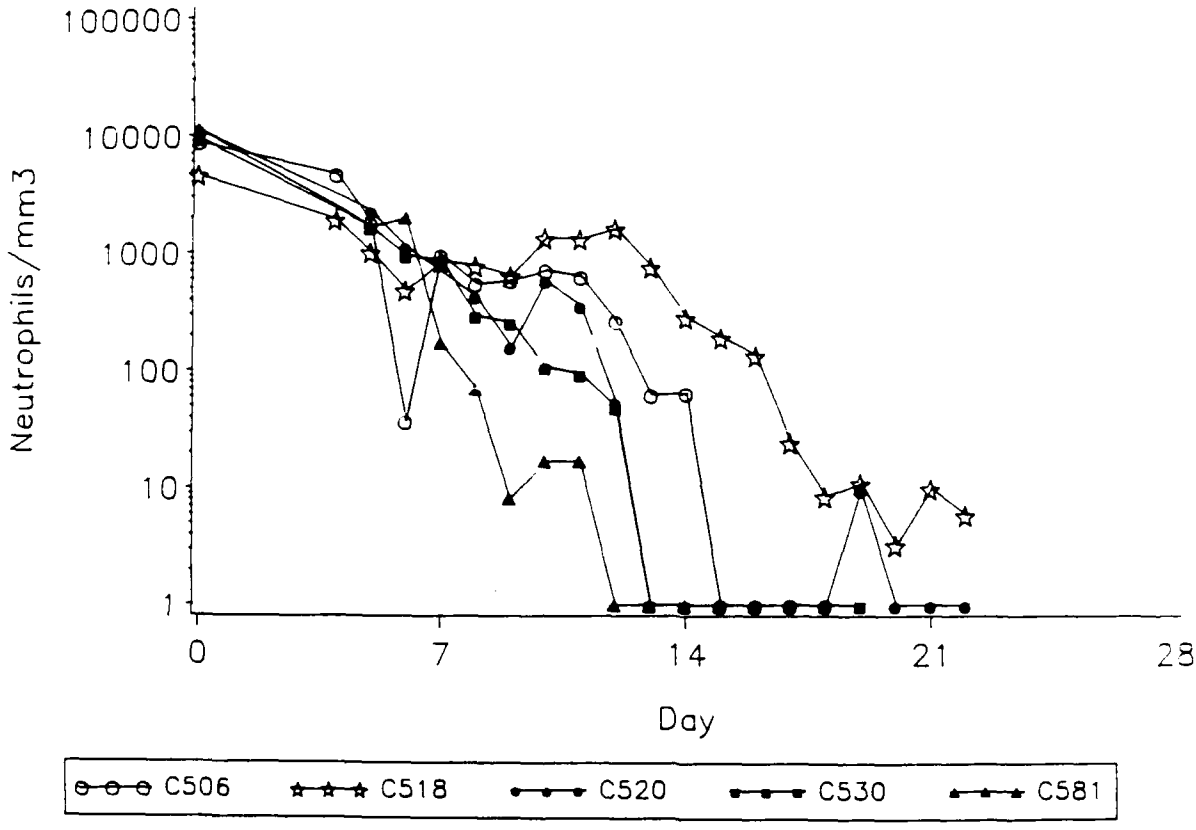

Figure 13. Peripheral blood neutrophil changes in dogs after 400 cGy of TBI (day 0).

Five other dogs were irradiated with 400 cGy TBI at 10 cGy per minute as before and, in addition, were treated with 100 μg rhG-CSF per kg/day subcutaneously twice a day for 21 days, starting within two hours after TBI. Four dogs showed complete and sustained hematopoietic recovery and are now surviving 120 to 180 days after TBI with normal peripheral blood counts (Figure 14). One dog, C726, died on day 19 after TBI because of accute bacterial pneumonia secondary to marrow aplasia. On the day of his death, peripheral blood neutrophil count was zero and marrow histology at autopsy showed 5% of normal marrow cellularity with few myeloid precursor cells but no erythroid or megakaryocytic precursor cells (Table 9).

A third group of five dogs was irradiated with 400 cGy TBI at 10 cGy per minute as before, and then treated with 100 μg rhG-CSF per kg/day subcutaneously twice a day from day 7 until day 20 after TBI or until death. All five dogs died between days 17 and 20 after TBI with infections secondary to marrow aplasia (Figure 15). In three dogs, marrow histology at autopsy showed no signs of hematopoiesis, and in two dogs 5% of normal marrow cellularity was seen containing myeloid precursor cells but no erythroid or megakaryocytic precursors (Table 9).

This study demonstrated that treatment with rhG-CSF allows sustained endogenous hematopoietic recovery in dogs after otherwise lethal TBI and also indicated that the time interval between TBI and start of rhG-CSF treatment is important.

After these encouraging results, a group of 10 dogs was irradiated with 500 cGy TBI at 10 cGy per minute and then treated with either 100 μg rhG-CSF per kg/day or 10 μg recombinant canine G-CSF (rcG-CSF) per kg/day subcutaneously twice a day for 21 days, starting within 2 hours after TBI. The dose of 10 μg rcG-CSF per kg/day was found to be equivalent biologically to the dose of 100 μg rhG-CSF per kg/day when given to normal dogs. Three dogs showed complete and sustained hematopoietic recovery and have been surviving for more than 90 days after TBI with normal peripheral blood counts (Figure 16).

6.3 EFFECT OF RECOMBINANT HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR ON HEMATOPOIETIC RECOVERY AFTER DLA-IDENTICAL LITTERMATE MARROW TRANSPLANTS IN DOGS.

Marrow transplant candidates either have no marrow function to begin with, as in aplastic anemia, or their marrow function is irradicated by the chemoradiotherapy aimed at destroying the underlying hematologic malignancy. Despite prompt infusion of HLA-identical marrow after completion of the conditioning regimen, patients remain pancytopenic for several weeks before the graft achieves full function. Typically, 15 to 44 (median 24) days will go by before granulocyte counts reach 1,000/mm^3 during which time patients are at risk for bacterial or fungal infections with an overall incidence of 20% to 25%, and a case mortality rate of 25% to 50%. Shortening the period of neutropenia after marrow grafting is likely to result in a reduction of the risk of infections. A

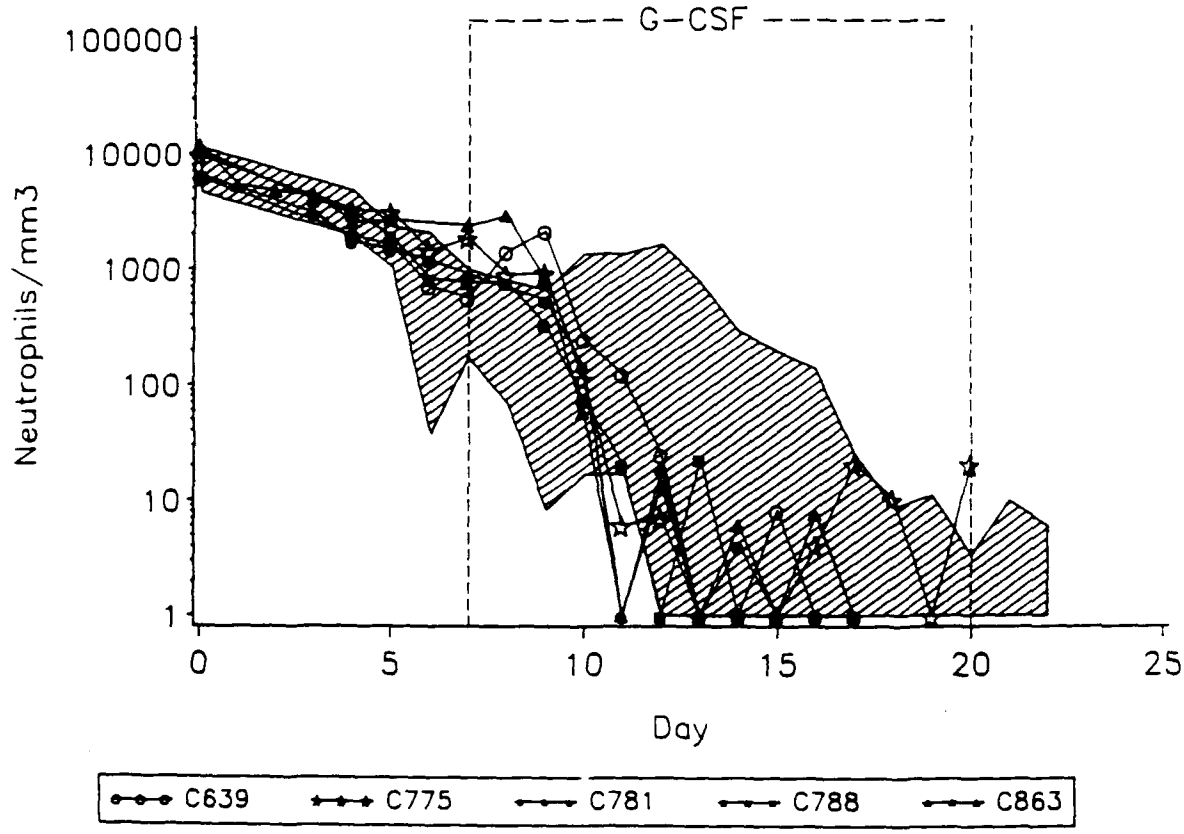

Figure 15. Peripheral blood neutrophil changes in dogs after 400 cGy of TBI (day 0) and twice-daily SQ injections of 100 μg rhG-CSF/kg/day from day 7 after TBI until death. Shaded area indicates the range of the peripheral blood neutrophils of the control dogs not receiving rhG-CSF after TBI.

preclinical study in nonhuman primates and two clinical studies using rhG-CSF after autologous marrow transplantation have shown significant acceleration of granulocyte recovery compared to the controls. The usefulness of rhG-CSF after allogeneic human marrow grafts has so far been investigated in only one non-randomized study, at least in part because of concerns of side effects such as an increased risk of graft-versus-host disease or late graft failure. We therefore studied the effect of rhG-CSF administered after transplantation of marrow from DLA-identical littermates in the dog model.

Table 10. Recovery of peripheral blood counts after TBI and transplantation of marrow from DLA-identical littermate without and with subsequent rhG-CSF.

Variable	Peripheral Blood Count	rh G-CSF	Number of Days to Achieve Specified Levels: 25% of Dogs	50% of Dogs	75% of Dogs	p-value[1]
Neutrophils	500/mm^3	-	8	10	15	.08
		+	6	7	7	
	1000/mm^3	-	11	14	16	.03
		+	7	8	8	
Monocytes	50/mm^3	-	15	28	35	.0008
		+	9	13	17	
	100/mm^3	-	29	49	105	.002
		+	13	17	22	
Lymphocytes	250/mm^3	-	10	15	22	.03
		+	8	9	13	
	500/mm^3	-	21	31	37	.01
		+	10	15	27	
Platelets	20,000/mm^3	-	13	20	37	.68
		+	22	26	31	

[1] Logrank test.

Table 10 and Figure 17 summarize the results on hemopoietic recovery. Neutrophil counts in the rhG-CSF treated dogs reached 500/mm^3 at a median of seven days and 1000/mm^3 at 8 days after transplantation. This was significantly faster than neutrophils recovery in the controls who reached values of 500 and 1000/mm^3 at

medians of 10 and 14 days, respectively (log rank test: $p<0.08$ and <0.03, respectively). The median time to reach 50 and 100 monocytes/mm^3 in G-CSF-treated dogs was 13 and 17 days compared to 28 and 49 days in the control group (log rank test: $p<0.0008$ and <0.002, respectively). Lymphocyte counts reached 250 and 500/mm^3 by day 9 and 15 in rhG-CSF-treated dogs compared to 15 and 31 days in the control dogs (log rank test: $p<0.03$ and <0.01, respectively). Platelet recovery to 20,000/mm^3 was not significantly different between the two groups (log rank test: $p<0.68$) with the median times being 20 days for the control and 26 days for the experimental group.

Table 11 summarizes the results on mortality, GVHD and graft failure. Three of ten G-CSF-treated dogs died before day 100 compared to five of fourteen control dogs (log rank test: $p=0.80$). Causes of death in the experimental group were GVHD (two dogs) and graft failure (one dog) and in the control group pneumonia (two dogs), graft failure (two dogs) and thrombocytopenic hemorrhage (one dog). Four of nine rhG-CSF-treated dogs with sustained engraftment developed persistent GVHD as documented by typical clinical symptoms (skin rash, diarrhea, jaundice) and histological findings at autopsy compared to one of twelve control dogs, but this difference was not statistically significant (two-tailed Fisher's exact test: $p=0.12$). Two of the four G-CSF-treated dogs with GVHD died because of this complication compared to none of the control dogs (two-tailed Fisher's exact test: $p=0.17$). One of ten rhG-CSF-treated dogs showed graft failure compared to two of fourteen control dogs (two-tailed Fisher's exact test: $p=1.00$). No unusual toxicities were seen in dogs receiving rhG-CSF.

Table 11. Mortality, GVHD, lethal GVHD and graft failure after TBI and transplantation of marrow from DLA-identical littermate without and with subsequent rhG-CSF.

	Dogs		
Variable	Control	rhG-CSF Treated	p-value
Death before 100 days (all causes)	5/14 (36%)	3/10 (30%)	.80[1]
GVHD[2]	1/12 (8%)	4/9 (44%)	.12[3]
Lethal GVHD	0/12 (0%)	2/9 (22%)	.17[3]
Graft Failure	2/14 (14%)	1/10 (10%)	1.00[3]

[1] Logrank test.
[2] The dogs which died of graft failure were not included in the comparison of incidence of GVHD and lethal GVHD.
[3] 2-tailed Fisher's exact test.

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