

Chronic Neutropenia

A New Canine Model Induced by Human Granulocyte Colony-stimulating Factor

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Abstract

Normal dogs were treated with recombinant human granulocyte colony-stimulating factor (rhG-CSF) at 10 µg/kg/day for 30 d, which caused an initial neutrophilia, followed by a prolonged period of chronic neutropenia. A control dog treated with recombinant canine G-CSF (rcG-CSF) showed persistent neutrophilia over 3 mo. Serum from dogs during neutropenia contained an antibody to rhG-CSF, which neutralized the stimulatory effects of both rhG-CSF and rcG-CSF on dog marrow neutrophilic progenitor cell growth and on NFS-60 cell proliferation. 4 mo after discontinuation of rhG-CSF, the dogs' neutrophil counts returned to the normal range. Rechallenge with the rhG-CSF re-induced severe neutropenia in 1 wk. Neutropenia was transferred by plasma infusion from a neutropenic dog to a previously normal dog. These data suggest that human rhG-CSF immunizes normal dogs and thereby induces neutralization of endogenous canine G-CSF and neutropenia. This model system should allow more precise definition of the *in vivo* role of G-CSF. (*J. Clin. Invest.* 1991; 87:704–710.) Key words: immunoglobulin • *in vitro* culture • *in vivo* treatment

Introduction

The recent cloning and expression of several hematopoietic growth factors have prompted a number of studies of their *in vivo* effects (1–8). The two most extensively studied factors, recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF)¹ and granulocyte colony-stimulating factor (rhG-CSF), have been found to ameliorate the neutropenia of chemotherapy and various marrow failure states (9–15). Much is known about the actions of these factors in *in vitro* systems (16, 17) where they were initially defined by their effects on proliferation of precursors of neutrophils and macrophages. In addition, both rhGM-CSF and rhG-CSF have been shown to activate mature leukocytes and to contribute to the inflammatory response system of the host (18, 19). However, their specific functions *in vivo* have not been defined. In the course of studies of the treatment of canine cyclic hematopoie-

sis with rhG-CSF (20), we also administered it to normal mongrel dogs. We observed that the animals developed severe chronic neutropenia and antibodies to rhG-CSF and rcG-CSF, results suggesting that G-CSF may play an important role in maintaining the normal blood neutrophil count.

Methods

Dogs and blood sampling. Normal mongrel dogs were housed in American Association for Laboratory Animal Care accredited animal care facilities as previously described (21–23). Periodic serum specimens were collected and daily blood counts were performed between 7 and 9 am before any treatment. Routine counts included a white blood cell count with differential, hematocrit, reticulocyte, and platelet determinations. Serial blood counts were obtained for 6–8 h following selected individual doses of G-CSF.

Bone marrow aspirations. Bone marrow samples were obtained before treatment and during neutropenia. 500 cell differential counts were performed on Wright-Giemsa-stained smears; data are presented as percentage of total nucleated cells.

Recombinant human and canine G-CSF. Purified rhG-CSF and recombinant canine G-CSF (rcG-CSF) were supplied by Amgen, Inc., (Thousand Oaks, CA). The rhG-CSF was administered subcutaneously once daily to two dogs for 30 d at 10 µg/kg/d. The rcG-CSF was administered to one dog at doses from 0.5 to 5 µg/kg/d for 4 mo. Dogs treated with rhG-CSF were subsequently given two additional doses of rhG-CSF separated by 1 wk (booster doses), 6 mo and 9 mo, respectively, after the initial treatment.

Immunoassay for antibodies to rhG-CSF and rcG-CSF. RIA and ELISA were used to assay for antibodies to rhG-CSF and cross-reactivities of antibodies to rhCSF and rcCSF. The assay for serum antibody to rhG-CSF was performed as follows. Using Immulon wells (Dynatech Laboratories Inc., Alexandria, VA), rhG-CSF was absorbed to the surface and unreacted binding sites blocked with BSA. Dilutions of the test and control sera of 1:10 or greater were then added to the wells for 2 h at room temperature. After washing thoroughly with PBS ¹²⁵I-labeled Staphylococcal-protein A (70–100 µCi/µg; New England Nuclear, Boston, MA) in 50 µl (≥ 100,000 cpm) was added. The wells were then washed again and radioactivity levels were determined. Bound radioactivity was graphed versus antibody dilution. Titer was defined as the serum dilution at which 50% of maximal specific counts bound was achieved. Control sera included rabbit anti-rhG-CSF (positive control), pre-bleed rabbit antiserum (negative control), and both dog and human control sera (negative controls, blank).

An ELISA assay was used to investigate the cross-reactivity of the antibodies in the dog serum to rhG-CSF and rcG-CSF. IgG were separated from sera from each of the dogs given subcutaneous rhG-CSF by using a Staphylococcal-protein A-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluting the column with glycine buffer at pH 2.3. After dialysis in PBS and determination of protein concentration, these samples were tested for binding to wells of Immulon plates previously coated with rhG-CSF or rcG-CSF. The IgG binding to the G-CSFs then was detected using horseradish peroxidase-conjugated staphylococcal-protein A (Amersham Corp., Amersham, UK) and orthophenylenediamine as the color reagent by standard techniques. Absorbance was read at 492 nm in a Titertek MCC/340 plate reader (Flow Laboratories, Melbourne, Australia).

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1. Abbreviations used in this paper: G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte/macrophage CSF; RabAb, rabbit antiserum; rc, recombinant canine; rh, recombinant human.

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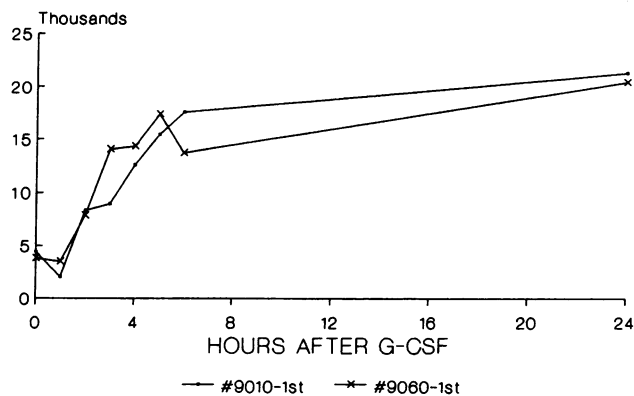


Figure 1. Neutrophils. Acute G-CSF response. Hourly blood neutrophil counts per microliter after the first doses of rhG-CSF in normal dogs (Nos. 9010 and 9060).

In vitro culture studies. In vitro bone marrow colony formation was used to assess the response of canine marrow cells to the growth factors as previously described (22–23). Bone marrow from anesthetized dogs was aspirated into heparin, diluted, and subjected to Ficoll-Hypaque density gradient centrifugation. The marrow cell population was then

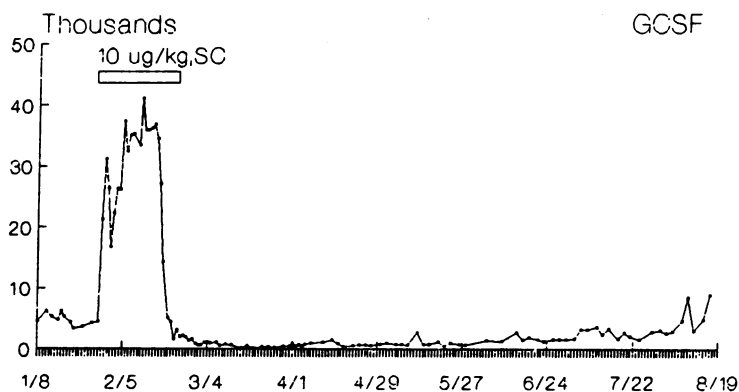
adhered to plastic T-75 flasks (Falcon Labware, Oxnard, CA) for 2 h at 37°C to deplete contaminating monocytes and reduce background in vitro colony formation. The mononuclear cells were plated at 10^5 /ml in the presence of 10% FCS and 3% normal dog serum obtained before administration of growth factor. rhG-CSF or rcG-CSF (56 or 282 pM) was used as the stimulus for colony formation. Post-endotoxin dog serum was used as the stimulant to estimate maximal colony forming capacity for each marrow specimen (23). Colonies (> 50 cells) and colony subtypes were counted at 8–10 d. To assay the capacity of treated dogs' sera to neutralize rhG-CSF or rcG-CSF, we substituted sera obtained after rhG-CSF administration for the normal dog serum in these cultures and assayed the effect of treated sera Ig fractions on colony formation stimulated by rcG-CSF or rhG-CSF. A rabbit polyclonal antiserum to rcG-CSF was used as a control for neutralization of added rhG-CSF or rcG-CSF.

The NFS-60 cell lines' (24) responsiveness to rhG-CSF was also used to assay the capacity of these dog sera to neutralize rhG-CSF bioactivity in vitro by a modification of previously published methods (25). The proliferation assay was carried out in 96-well microtiter plates containing serial dilutions of test sera (IgG fractions), a quantity of rhG-CSF giving 70–80% of maximal stimulation and 50 μ l of NFS-60 cells (10^6 cells per ml). Cultures were incubated for 24 h at 37°C, after which 0.5 μ Ci of [3 H]thymidine (5 Ci/mmol, Amersham Corp.) was added, it was incubated another 6 h, cells were harvested, and incorporated radioactivity was measured. A murine monoclonal anti-rhGM-CSF antibody (LMM111) (26) was used as a negative control.

NEUTROPHILS

9010

GCSF



NEUTROPHILS

9060

GCSF

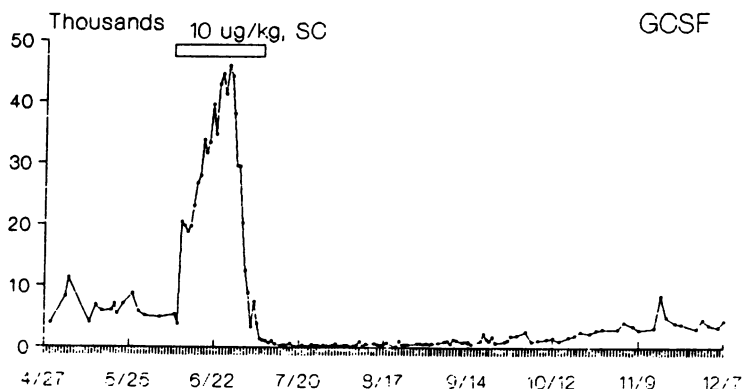


Figure 2. Serial blood neutrophil counts before, during, and after rhG-CSF administration (box) in the same dogs as in Fig. 1.

NEUTROPHILS

11717

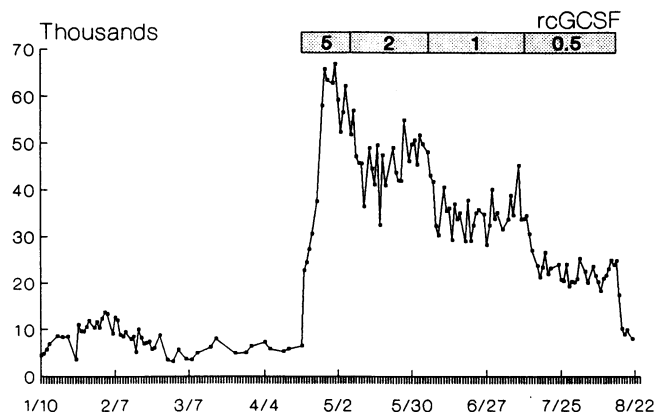


Figure 3. Serial neutrophil counts during rcG-CSF administration in normal dog 11717. G-CSF dose varied from 0.5 to 5.0 $\mu\text{g/kg/d}$ as indicated in the shaded bars. The rcG-CSF was stopped on 8/16 and the counts quickly returned to baseline.

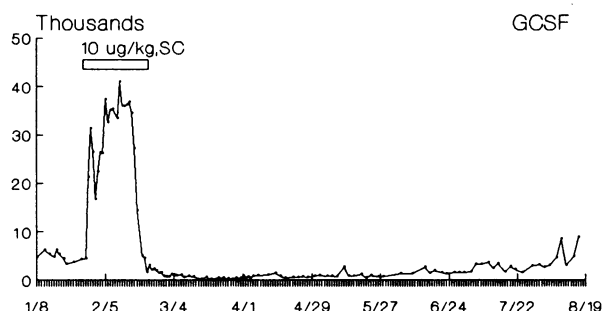
Plasma reinfusion. Blood was collected on two separate days from a dog 2 wk after the booster injections and anticoagulated with acid-citrate-dextrose. Plasma was separated, frozen, and subsequently transfused to a healthy normal dog. The plasma was given in two doses in 2 d; a total dose of 32 ml/kg body weight was given. Blood counts were then followed for the next 30 d.

Results

The effect of G-CSF on blood and bone marrow counts. The effects of the first rhG-CSF injections in each dog demonstrate an initial slight fall in the first hour followed by a sustained rise in circulating neutrophil counts (Fig. 1). On the 5th and 10th days after therapy began, pre-injection counts were increased and serial follow-up counts again showed this "dip-and-rise" pattern. The daily pretreatment neutrophil counts demonstrate a chronic, stable neutrophilia over the first 18–21 d. Then an abrupt decline to baseline and below occurred (Fig. 2). Despite discontinuation of rhG-CSF injections after day 30, the severe neutropenia persisted for months and then gradually

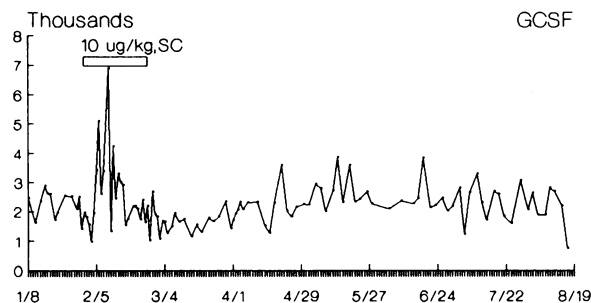
NEUTROPHILS

9010



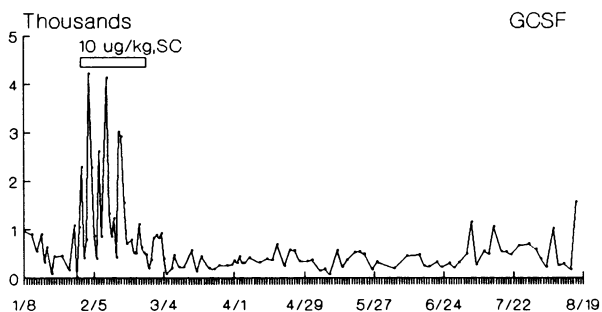
LYMPHOCYTES

9010



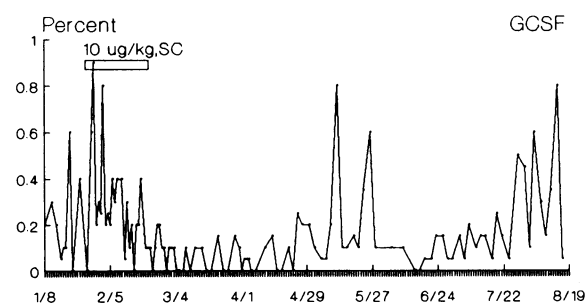
MONOCYTES

GCSF



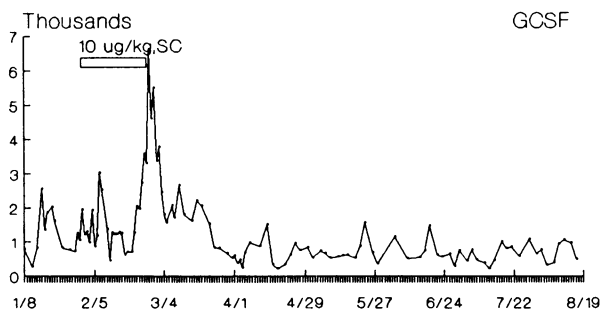
RETICULOCYTES

GCSF



EOSINOPHILS

GCSF



PLATELETS

GCSF

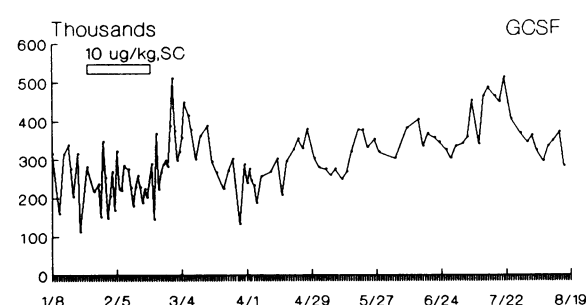


Figure 4. Serial blood counts for neutrophils, monocytes, eosinophils, lymphocytes, reticulocytes, and platelets in dog 9010 over the same period as in Fig. 2.

Table I. Bone Marrow Differential Counts

Cells	Dog:	Before		During		After	
		9010	9060	9010	9060	9010	9060
Neutrophilic precursors							
Mitotic		9.2	9.0	19.8	25.8	7.0	8.6
Postmitotic		38.2	32.2	52.4	43.4	39.4	21.4
Eosinophils		4.6	3.8	2.6	2.2	9.2	3.8
Basophils		0.2	0	0	0.2	1.2	0
Monocytes		4.0	1.2	1.8	0.6	2.8	0.6
Erythroid precursors		28.8	38.8	15.0	23.4	27.6	49.0
Lymphocytes		13.6	12.0	7.0	3.8	10.4	13.8
Plasma cells		1.0	2.0	1.0	0.4	2.4	2.8
Others		0.4	1.0	0.4	0.2	0	0
PM/M ratio*		4.15	3.58	2.65	1.68	5.62	2.49
M/E ratio†		1.65	1.06	4.81	2.96	1.68	0.61

Marrows obtained before rhG-CSF, during rhG-CSF, at peak neutrophilia, and after rhG-CSF during neutropenia. * Ratio of postmitotic neutrophils (metamyelocytes plus bands plus neutrophils) to mitotic neutrophils (myeloblasts plus promyelocytes plus myelocytes). † Ratio of myeloid to erythroid precursors.

remitted. The neutrophil count fell to the range of 100–500/ μ l by 4 wk after stopping the rhG-CSF and at 12 wk remained low in the range of 800–1200/ μ l. In contrast, a dog given rcG-CSF demonstrated a marked and sustained neutrophilia (Fig. 3); within 4 d after discontinuing the rcG-CSF, the neutrophil count had returned to the normal range and did not fall below normal.

The other blood cell counts demonstrate the relative selectivity of the effect of rhG-CSF on blood neutrophils (Fig. 4). There was a slight rise in monocyte counts during the neutrophilia induced by the rhG-CSF and a burst of eosinophilia as the neutrophil count was falling, but mean pretreatment (30 d before G-CSF) counts of monocytes, lymphocytes, eosinophils, and platelets were not different from post-treatment (from days 60 to 90 after start of G-CSF) counts when stable, severe neutropenia was present.

Bone marrow differential counts performed on 500 cells from marrows before and during neutropenia show some decrease in proportions of neutrophilic precursors (Table I). However, all stages of neutrophilic maturation were evident in neutropenic dogs' marrows.

Table II. Serum Antibodies to rhG-CSF*

Week	Dog 9010	Dog 9060
Before	1:50	1:50
1	—	1:43
2	1:40	1:40
3	1:300	1:220
4	1:1,000	1:500
6	—	1:700
10	1:200	1:150
14	1:36	1:65
52	1:40	—

* Dilution titers at which 50% of maximal cpm demonstrated. Control normal dog serum gave background titer of 1:50.

Antibody studies. When antibody to rhG-CSF was measured, the pretreatment sera had no antibody whereas between day 14 and 21 antibody titer rose dramatically (Table II). The level of antibody remained elevated at least 2 mo after discontinuation of the rhG-CSF.

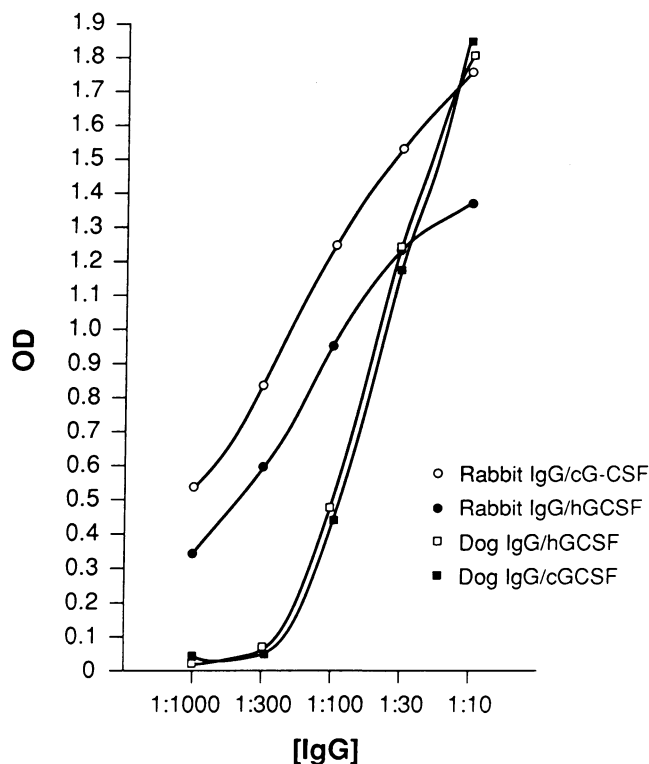
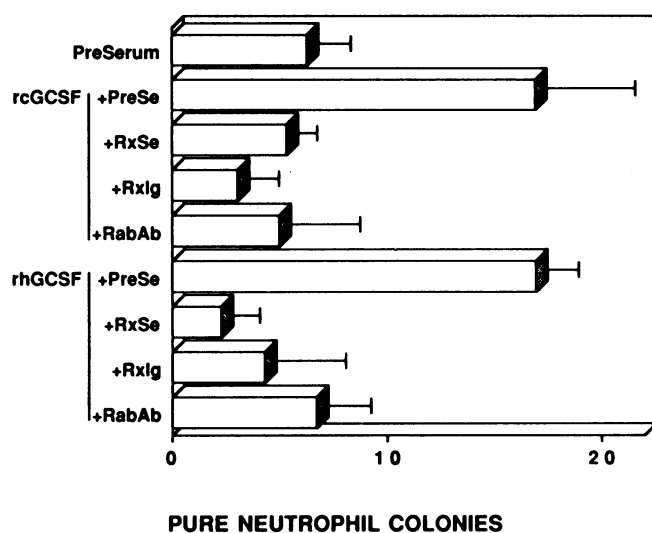


Figure 5. The reactivity of IgG purified from dog 9010 and a rabbit immunized with rhG-CSF. Antibody bound to wells coated with 10 ng G-CSF was measured by ELISA as a function of dilution of IgG fraction (OD: optical density at 492 nm). The protein concentration of the purified dog and rabbit IgGs were similar (stock solutions ~ 1 mg/ml). Similar results were found on the second dog.



PURE NEUTROPHIL COLONIES

Figure 6. Neutrophilic colony formation by 10^5 nonadherent dog marrow mononuclear cells is plotted ± 1 SD. When stimulated by 3% serum obtained before any treatment (preserum) background colony formation was seen; with rcG-CSF or rhG-CSF (rcG-CSF + PreSe or rhG-CSF + PreSe), colonies increased. With added rcG-CSF or rhG-CSF plus dog Ig from treated dog (RxIg) or rabbit Ig to rcG-CSF (RabAb) or when treated dog serum was substituted for PreSerum (RxSe), colony formation returned to background. Maximal colony formation in this experiment was, for neutrophil colonies, $22 \pm 4/10^5$ cells, and for total colonies, $113 \pm 10/10^5$ cells.

IgG fractions from the dogs' plasma were tested for their reactivity with rhG-CSF and rcG-CSF in the ELISA assay. Rabbit IgG fractions from animals immunized with rhG-CSF were compared with the dog samples. For both dogs given the rhG-CSF, the IgG was equally reactive with rhG-CSF and rcG-CSF. The rabbit anti-rhG-CSF antibody also reacted with the G-CSF of both species (Fig. 5).

In vitro culture studies. Dog bone marrow cell culture studies demonstrated that sera obtained from the neutropenic phase (RxSe) blocked neutrophil colony formation by nonadherent marrow cells induced by 3% normal dog serum preserum plus rhG-CSF or rcG-CSF (Fig. 6). Background colony

formation was noted even in the presence of a neutralizing rabbit antiserum (RabAb) to rcG-CSF. Immunoglobulin extracted from the serum of one of the neutropenic dogs (RxIg) added to 3% normal dog serum blocked the stimulated increase in neutrophil colonies induced by either rhG-CSF or rcG-CSF (Fig. 6). Macrophage colonies were not altered by the dog sera or rabbit antibody to rcG-CSF (data not shown).

In the NFS-60 assay, the dog antibodies were tested for their capacity to neutralize rhG-CSF and rcG-CSF bioactivity. In these experiments the dog antibody fractions were compared with rabbit anti-rhG-CSF and murine anti-rhGM-CSF MABs. The Ig fractions from sera of dogs 9010 and 9060 (post-G-CSF) at $100 \mu\text{g/ml}$ completely inhibited the NFS-60 cell response to rhG-CSF, as did the rabbit anti-rhG-CSF fraction (Fig. 7 A). Half-maximal inhibition was obtained with $2 \mu\text{g/ml}$ 9010 dog IgG, $9 \mu\text{g/ml}$ 9060 dog IgG, and $2 \mu\text{g/ml}$ rabbit IgG. In cultures stimulated with rcG-CSF, the anti-G-CSF antibodies were inhibitory, although not as active as against rhG-CSF (Fig. 7 B). Half-maximal inhibition was achieved with $30 \mu\text{g/ml}$ 9010 dog IgG, $> 100 \mu\text{g/ml}$ 9060 dog IgG, and $20 \mu\text{g/ml}$ rabbit IgG. The anti-rhGM-CSF antibody had no effect.

Re-challenge and plasma infusion studies. After each animal had recovered from neutropenia, two booster doses of rhG-CSF, $10 \mu\text{g/kg/dose}$, separated by one week were administered and sequential neutrophil counts following each dose were determined (Fig. 8). The neutrophil count increases after the first booster dose appeared similar to those recorded initially (Fig. 1). However, by 1 wk later, the pretreatment counts were low and failed to show any increment in response to the rhG-CSF.

Plasma obtained from dog 9010 after rechallenge (when the antibody titer was elevated to between 1:250 and 1:500) was infused into a naive dog [11717] and within 3 d caused a significant fall in neutrophil counts (Fig. 9). The decreased counts persisted for a few weeks and remitted. The neutropenia was not associated with obvious changes in other cell counts.

Discussion

The development of neutropenia by two dogs after administration of rhG-CSF for 30 d was associated with the development

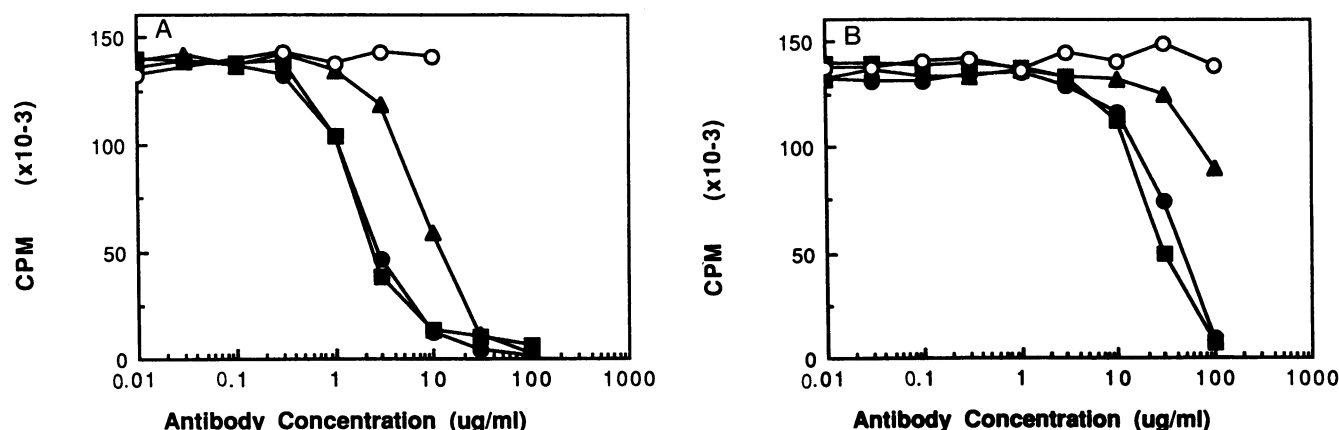


Figure 7. Inhibition of the NFS-60 response to G-CSF by IgG fractions of dog sera taken during neutropenia. (A) Response to rhG-CSF; (B) response to rcG-CSF. Cells were cultured with 0.5 ng/ml G-CSF and serial dilutions of antibodies from $100 \mu\text{g/ml}$. (●) Dog 9010; (▲) Dog 9060; (■) rabbit anti-G-CSF; (○) LMM111 (anti-GM-CSF). Data points are the means of triplicate cultures. Standard deviations were $< 10\%$ of means.

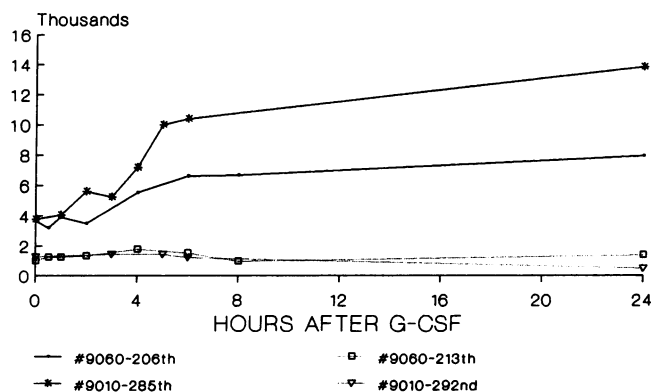


Figure 8. Neutrophils. Acute G-CSF response. Hourly blood neutrophil counts after the two rechallenge doses of rhG-CSF in each dog. The dog numbers are followed by the number of days after the first dose of rhG-CSF for that dog. Closed symbols are for the first dose and open symbols for the second rechallenge dose.

of antibody to rhG-CSF. We then demonstrated that this antibody was cross-reactive with rcG-CSF and that it neutralized both rhG-CSF and rcG-CSF activities in vitro. The rapid recurrence of neutropenia after rechallenge would be consistent with an anamnestic antibody response. The transfer of neutropenia by plasma confirms the humoral basis for the neutropenia. These data suggest that elimination of endogenous G-CSF action can be a mechanism of neutropenia and that G-CSF is an important regulator of the level of blood neutrophil counts.

The appearance of cytopenias after administration of heterologous hematopoietic growth factors was first reported at least 25 yr ago (27). In classical experiments done in rabbits, investigators attempted to elicit high titer antibodies to human erythropoietin, and observed that those animals developing high antibody titers became anemic. Evidence then suggested that it was indeed the antibody to the human factor that interfered with endogenous red cell production, via inhibition of rabbit erythropoietin (28, 29). Whether the syndrome of pure red cell aplasia can be caused by a similar antibody to erythropoietin remains uncertain (30).

PLASMA INFUSION

11711

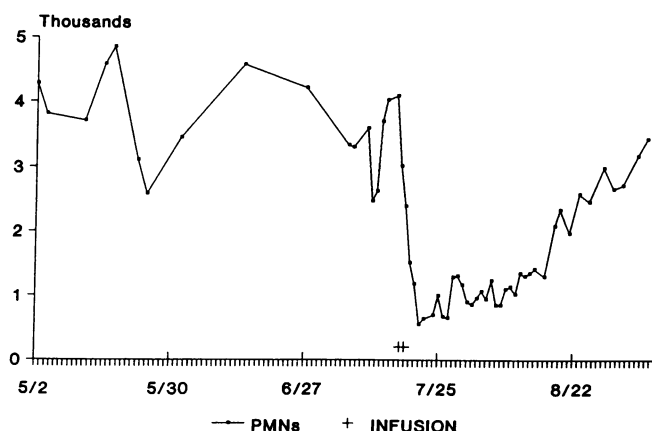


Figure 9. Serial blood neutrophil counts after infusion of plasma from dog 9010 into dog 11711 (32 ml/kg) indicated by a plus sign.

The present data demonstrate that chronic administration of human G-CSF induces neutropenia in dogs with previously normal neutrophil counts. Interestingly, the other cell counts were not reduced. The bone marrow differential counts showed mild changes and did not demonstrate a clearcut mechanism for neutropenia. Importantly, there was no apparent decrease in precursors for other marrow-derived cells, although quantitative studies were not performed. Although the blood neutrophil counts were low, they were not sufficiently low to anticipate illness in the dogs and none was observed.

Classically, "immune" forms of neutropenia occur when an antibody recognizes an antigenic determinant on the surface of the neutrophil, leading to premature removal of circulating cells (31). However, in this case we have hypothesized that administration of human G-CSF elicited an antibody capable of neutralizing human G-CSF that cross-reacted with canine G-CSF and induced a state of "G-CSF deficiency." It is also possible that the antibody to human G-CSF bound to canine G-CSF not only in body fluids but also on the surface of the neutrophil, resulting in premature destruction of mature neutrophils and a variant of the classical type of antibody-mediated neutropenia (31). The plasma infusion studies, however, argue against this concept. If the latter mechanism occurred, we would have expected a more abrupt fall in the counts than was observed, as well as a more rapid recovery. Only detailed neutrophil kinetic studies will separate these possible mechanisms.

This neutropenia in dogs may thus represent a novel "auto-immune" mechanism for neutropenia in man. As a new model, further study may allow elucidation of the normal physiologic role(s) of G-CSF and its relationship to other CSFs in the regulation of normal granulocytopoiesis. The understanding of these relationships should permit definition of the pathophysiologic mechanisms causing both neutrophilia and neutropenia, leading to more effective strategies for management of patients with these disorders.

Acknowledgments

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