A Comparison of Treatment of Canine Cyclic Hematopoiesis With Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), G-CSF, Interleukin-3, and Canine G-CSF

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Cyclic hematopoiesis in gray collie dogs is a stem cell disease in which abnormal regulation of cell production in the bone marrow causes cyclic fluctuations of blood cell counts. In vitro studies demonstrated that recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and granulocyte colony stimulating factor (G-CSF) all stimulated increases in colony formation by canine bone marrow progenitor cells. Based on these results, gray collie dogs were then treated with recombinant human (rh) GM-CSF, IL-3, or G-CSF subcutaneously to test the hypothesis that pharmacologic doses of one of these hematopoietic growth factors could alter cyclic production of cells. When recombinant canine G-CSF became available, it was tested over a range of doses. In vivo rhIL-3 had no effect on the recurrent neutropenia but was associated with eosinophilia. rhGM-CSF caused neutrophilia and eosinophilia but cycling of hematopoiesis persisted. However, rhG-CSF caused neutropenia, prevented the recurrent neutropenia and, in the two animals not developing antibodies to rhG-CSF, obliterated periodic fluctuation of monocyte, eosinophil, reticulocyte, and platelet counts. Recombinant canine G-CSF increased the nadir neutrophil counts and amplitude of fluctuations at low doses (1 μg/kg/d) and eliminated all cycling of cell counts at high doses (5 and 10 μg/kg/d). These data suggest significant differences in the actions of these growth factors and imply a critical role for G-CSF in the homeostatic regulation of hematopoiesis.

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CYCLIC HEMATOPOIESIS is a rare disease that occurs both in humans and gray collie dogs. In this disorder, neutrophils and monocytes cycle with extraordinary regularity; in most patients as well as the dogs, reticulocytes, platelets, eosinophils, and lymphocytes also cycle with periodicity identical to that of the neutrophils. The disease is transferable by bone marrow transplantation, suggesting a disorder of the hematopoietic stem cell in which a periodic failure of cell production is the proximate cause of the neutropenia. Based on the highly predictable cyclic fluctuations of the counts, several mathematical models for the disorder have been proposed. In each of the models, some form of disordered feedback regulation has been proposed, although the precise biochemical mechanism(s) underlying such feedback have not been defined.

The recent cloning of several growth factors for cells in the hematopoietic system has prompted a number of in vivo studies of the efficacy of these factors to ameliorate the neutropenia of chemotherapy or various marrow failure states. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are both capable of stimulating the progenitor cells for erythrocytes and platelets, as well as granulocytic cells, and thus seemed good candidates for the feedback stimulator that might be deficient in cyclic hematopoiesis. Similarly, G-CSF acts both at early and late stages of granulocytopenia and could be defective in this disease. Therefore, we compared the effects of recombinant human (rh) IL-3, GM-CSF, and G-CSF and, when it became available, recombinant canine G-CSF (rcG-CSF) on hematopoiesis in gray collie dogs. Since our initial report on the effects of rhGM-CSF, Lothrop et al. reported on the response of cyclic neutropenic dogs to rhG-CSF as well as rhGM-CSF. Our data extend that report by showing in vitro responsiveness of canine progenitor cells to all three recombinant human hematopoietic growth factors as well as rcG-CSF. They also illustrate that the in vivo alterations in cell counts are different for each specific growth factor.

MATERIALS AND METHODS

Dog and blood sampling. Male gray collie dogs (ages 1 to 4 years) and normal mongrel dogs were housed in AAALAC (American Association for Accreditation of Laboratory Animal Care) accredited animal care facilities as previously described. Daily blood counts were performed between 7 AM and 9 AM, including a white blood cell count with differential, hematocrit, reticulocyte, and platelet determinations.

In vitro colony formation assays. In vitro bone marrow colony formation was used to assess the potential responsiveness of canine marrow cells to the growth factors. Assays were performed as previously described using normal canine bone marrow. Bone marrow was aspirated into heparin, diluted, and subjected to Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation. To reduce background growth factor production by cells in the marrow, two cycles of adherence to the surface of plastic flasks (Falcon, T-75, Lincoln Park, NJ) for 1 hour at 37°C were performed. The mononuclear cells were plated at 1 x 10⁶ cells/mL in the presence of 10% fetal calf serum, 5% normal dog serum, and varying concentrations of exogenous growth factors. Colonies (greater than 50 cells) were counted at 8 to 10 days ("total colonies"). As a positive control, phytohemagglutinin-stimulated canine leukocyte conditioned medium (PHA-LCM) was routinely assayed as an

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estimate of maximal colony-forming capacity for each marrow specimen.

**Hematopoietic growth factors.** Purified rhGM-CSF, provided by Genetics Institute, Inc (Cambridge, MA) was added to in vitro cultures at final concentrations ranging from 1 pmol/L to 5 pmol/L. It also was administered by subcutaneous injection to three dogs every 8 hours for up to 28 days, one each at total doses of 5, 30, and 50 µg/kg/d.

Purified rIL-3, rhG-CSF, and rcG-CSF provided by AMGen, Inc (Thousand Oaks, CA) were similarly added to in vitro cultures at final concentrations from 0.1 pmol/L to 160 pmol/L for IL-3 and 1.4 pmol/L to 564 pmol/L for G-CSF. IL-3 and rhG-CSF were administered subcutaneously once daily to 2 and 3 dogs, respectively, for either 30 or 60 days at 10 µg/kg/d. Both rhIL-3 and rhG-CSF were administered concurrently to two dogs for 30 days at 10 µg/kg/d for each drug. rcG-CSF was administered to two dogs for over 4 months at doses between 1 and 10 µg/kg/d.

**Antibody assays.** An enzyme-linked immunosorbent assay (ELISA) for canine antibody to rhGM-CSF was performed on serum specimens obtained weekly beginning before treatment and continuing for 6 weeks by a modification of previously published methods. Dynatech Immulon-2 plates were coated with CHO expressed GM-CSF at 30 ng/well. Plates were blocked with 3% gelatin in phosphate-buffered saline (PBS). Serum samples were diluted 10-fold serially in PBS containing 0.05% Tween 20. Diluted samples were added to the plates at 30 µL/well in triplicate and incubated overnight at 4°C. The following day, plates were washed three times with PBS and 0.05% Tween 20. Affinity-purified rabbit anti-dog immunoglobulin G (IgG) (heavy and light chain) linked to alkaline phosphatase (Zymed lot 50430, South San Francisco, CA) was diluted 1:1,000 in PBS and 0.05% Tween 20, and added at 30 µL/well for 2 hours at 37°C. Plates were washed three times in PBS containing Tween-20 and substrate added consisting of 1X Sigma phosphatase substrate tablet (5 mg) in 5 mL diethanolamine buffer and added at 30 µL/well. The plates were then read at 410 nm and the results expressed as the titer of serum at which 50% maximal binding occurred. Samples were coded and assayed without knowledge of treatment status.

A radioimmunoassay was developed to measure potential antibodies to rhG-CSF in sera using a solid-phase binding format. In this assay, rhG-CSF is absorbed to the surface of Immulon wells (Dynatech, Chantilly, VA), and unreacted protein binding sites are blocked with bovine serum albumin. Dilutions (1:10 or greater) of sera to be tested are then incubated in the wells. Control sera included in this assay are rabbit anti-rhG-CSF (positive control), pre-bleed rabbit antiserum (negative control), and human control serum (negative control, blank). The wells were washed and incubated with 100,000 cpm of 125I-protein A (New England Nuclear, Boston, MA; 70 to 100 µCi/µg). The wells were washed again and radioactivity levels determined. Bound radioactivity was graphed versus antibody dilution. Titer was defined as the serum dilution at which 50% maximal specific counts bound was achieved.

**RESULTS**

**In vitro colony formation.** Normal dog bone marrow cells responded to each of the recombinant human hematopoietic growth factors with increased colony formation (Fig 1). The plateau for increased colony numbers (per 10⁶ nonadherent marrow mononuclear cells) was achieved at 56 pmol/L for rhG-CSF, 56 pmol/L for rhGM-CSF, 1 pmol/L for rhIL-3 (note log scales), and 112 pmol/L for rcG-CSF. These increases in colony numbers represented between 41% and 106% of the maximal increases generated by dog PHA-stimulated leukocyte-conditioned medium.
**In vivo effects.** The recombinant human growth factors each produced a different alteration of cell counts. The rhIL-3 produced the least effect (Figs 2 and 3). In both dogs treated with rhIL-3 alone, the initial peak in neutrophil counts appeared to be higher than previously, but in the second treated dog (Fig 3) subsequent peaks of corresponding size were present in association with clinical infections. The regularly recurrent neutropenia was not altered by treatment with rhIL-3; however, an increase in eosinophil counts was evident, suggesting in vivo activity similar to that seen in primates.40

The administration of rhGM-CSF increased the neutrophil, monocyte, and eosinophil counts (Figs 4 and 5). Of particular note, marked neutrophilia (in excess of $40 \times 10^9/\mu L$) and a sustained monocytosis occurred. The eosinophil count began to increase approximately 2 weeks after the initiation of therapy and peaked at very elevated levels. The neutropenic nadirs recurred despite treatment. After 3 weeks
of treatment both dogs failed to mount neutrophilia despite continued therapy. In the second dog the platelet count showed an initial decrease and then resumed cycling, but at a lower level than previously. The dog treated at 5 μg/kg/d showed essentially no change in any cell counts (data not shown); a normal dog treated with rhGM-CSF at 50 μg/kg/d showed a two- to fourfold increase in neutrophil count over a 30-day treatment course (data not shown), findings comparable with recently published data.¹⁴

In striking contrast, rhG-CSF treatment caused a prominent neutrophilia that was sustained for at least 20 days in all five treated dogs. Of note, three dogs stopped responding after 20 to 25 days and resumed cycling despite continued therapy, precisely as reported by Lothrop et al¹⁴ (data not shown). Two of these three dogs were given simultaneous treatment courses of both rhIL-3 and rhG-CSF; we observed no difference in count responses comparing the dogs receiving concurrent rhG-CSF and rhIL-3 with those receiving rhG-CSF alone. However, two dogs showed persistent responses to rhG-CSF lasting 60 days (Figs 6 and 7). In these two animals, the abrogation of apparent cyclical neutrophil count fluctuations was associated with loss of regular fluctuations in the other cell counts. This was most pronounced in the platelet counts (see Figs 6 and 7). When rhG-CSF was
administered, we observed abrogation of all cycling at doses of 5 to 10 μg/kg/d similar to that observed with rhG-CSF (Figs 8 and 9). At a lower dose of 1 μg/kg/d, we observed a return of cyclic variation, with higher nadirs and a slightly shorter cycle period.

Dog antibody. The assays for antibody to rhGM-CSF and rhG-CSF demonstrated no antibody before treatment. In the dog treated with rhGM-CSF at 50 μg/kg/d, the initial increase in titer to 1:200 occurred on day 12 with a subsequent peak at 1:6,000 on day 26 (Table 1). A similar pattern of antibody titer was documented in the other two dogs receiving 5 and 30 μg/kg/d, respectively (data not shown). In the dogs treated with rhG-CSF, the antibody was detected by 2 to 4 weeks in all animals. However, the two dogs with persistent responses to the rhG-CSF had antibody titers of 1:200 or less on all measurements.

DISCUSSION

Canine cyclic hematopoiesis is a rare disorder in which two therapies, endotoxin and lithium, are known to alter cycling. Both agents are believed to act, at least in part, via stimulation of endogenous production of CSF. We hypothesized that administration of a purified recombinant growth factor might also ameliorate this disorder. The availability of purified recombinant hematopoietic growth factors offered us the opportunity to test this hypothesis directly.

The data presented here demonstrate that all three recombinant human growth factors as well as the recombinant canine G-CSF stimulate granulocytopenesis in dogs.

![Fig 4. Serial blood counts on gray collie no. 9298 during rhGM-CSF treatment at 50 μg/kg/d as indicated by the stippled area. This rhGM-CSF treatment course occurred 10 months before the period illustrated in Fig 3.](image1)

![Fig 5. Serial blood counts on gray collie no. 8027 during rhGM-CSF treatment at 30 μg/kg/d shown by stippled area. Note dip in platelet counts during treatment.](image2)
conclusion is supported both by the in vitro data and by the increase in peripheral blood leukocyte counts in the dogs. The increases in eosinophil counts (for rhIL-3 and rhGM-CSF), monocyte counts (for rhGM-CSF), and neutrophil counts (rhGM-CSF, and both rhG-CSF and rcG-CSF) were to levels rarely seen in our extensive prior experience with these animals. This suggests significant homology between the hematopoietic growth factors of dogs and humans.

Our report is in agreement with Lothrop et al.\textsuperscript{14} that rhGM-CSF does not appear to alter the underlying pathophysiologic events producing cyclic hematopoiesis. Lothrop et al.\textsuperscript{14} noted the failure of rhGM-CSF to cause neutrophilia, which could have been due to the low-dose used; at the same doses (5 μg/kg/d), neutrophilia was observed in neither study, but occurred in both dogs that we treated with higher doses. Our data confirm their finding that rhG-CSF prevents the recurrent neutropenia, and extend it by showing that in some animals the neutrophilia could be sustained for months and that fluctuations in other cell types were blunted in dogs with continuing responses to rhG-CSF and rcG-CSF.
Fig 7. Serial blood counts on gray collie no. 8027 during rhG-CSF treatment at 10 μg/kg/d for 60 days. This period followed that shown in Fig 5 by 11 months.

data on rhIL-3 suggest that this factor may be active in stimulating marrow cell growth but has a limited effect on mature blood cell counts.

Dogs developed antibodies to the rhGM-CSF or rhG-CSF in 2 to 3 weeks, and soon thereafter their response to the factor was lost. It seems likely that these antibodies neutralized the in vivo effects of the exogenous growth factors. The two dogs with persisting response to rhG-CSF had the lowest antibody titers, increasing the probability that this is a causal relationship. All three of the dogs originally treated with rhGM-CSF (all of whom developed antibodies to rhGM-CSF) were subsequently treated with rhG-CSF; two developed antibody to rhG-CSF whereas the other did not. This suggests a difference in canine antibody responses to these two human hematopoietic growth factors, potentially reflecting differences in the degree of homology between the canine and human factors.

Eosinophilia developed late during therapy with rhGM-CSF, possibly as a direct effect of the rhGM-CSF or as an immunologic reaction to it. The latter mechanism is suggested since the dogs developing antibodies to rhG-CSF also developed eosinophilia.
Fig 8. Serial blood counts on gray collie no. 11665 during rcG-CSF treatment at doses of 5, 10, 1, and 2 μg/kg/d.

The platelet count decreased with rhGM-CSF in one treated dog, while both rhG-CSF and rcG-CSF stabilized the platelet count in the normal range. A decrease in platelet count was seen using rhGM-CSF from a different source in normal dogs in a bone marrow transplantation model system. These data suggest that rhGM-CSF has generalized effects on multiple cell counts in dogs, but that G-CSF may act at a locus closer to the defect causing canine cyclic hematopoiesis.

The data reported here with rcG-CSF constitute the initial report of efficacy of the canine factor for cyclic hematopoiesis. The ability of this factor to maintain stimulation of neutrophil counts over months and the observation that cycling of all cell counts apparently stops during therapy at 5 to 10 μg/kg/d suggest a profound effect on the regulatory system. Further, the appearance of recurrent cycling at the reduced rcG-CSF dose of 1 μg/kg/d, with shortening of the cycle length, closely resembles our recent findings in patients with congenital cyclic hematopoiesis. In that report we noted continued cycling of all counts, with reduction in period length from 21 to 14 days, associated with reduction in both infection frequency and severity. Hence, these data...
strongly support the established concept that the canine disease is a close analogue of the human disorder, with similar therapeutic responsiveness as well. We speculate that if we were able to administer higher doses of rhG-CSF to patients, we might see in them a similar elimination of cycling. Finally, these data suggest very important differences exist in the mechanisms of action of the hematopoietic growth factors. It appears likely that elucidation of the specific defect in cyclic hematopoiesis will reveal important information about the physiology and cellular anatomy of homeostatic mechanisms in hematopoietic regulation.

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