Correction of Canine Cyclic Hematopoiesis With Recombinant Human Granulocyte Colony–Stimulating Factor

By Clinton D. Lothrop, Jr, David J. Warren, Lawrence M. Souza, J.B. Jones, and Malcolm A.S. Moore

Canine cyclic hematopoiesis (CH) is an autosomal recessive disease of gray collie dogs that is characterized by neutropenic episodes at 14-day intervals. The biochemical basis for CH is not known but may involve a regulatory defect of the response to or production of a hematopoietic growth factor. Administration of recombinant human granulocyte colony–stimulating factor (rhG-CSF) to two CH dogs and one normal dog caused a marked leukocytosis (>50,000 WBCs) in all three dogs. The leukocytosis was due largely to a greater than tenfold increase in neutrophils. Less pronounced but significant elevations in monocytes occurred during G-CSF treatment. The elevated WBC count was maintained for more than 20 days in all three dogs, and two predicted neutropenic episodes were prevented in both CH dogs during rhG-CSF treatment. A decline in the WBC count occurred simultaneously in all three dogs during the last five treatment days and was presumably associated with the development of neutralizing antibodies to the heterologous rhG-CSF protein. Bone marrow evaluation indicated that the swings in the myeloid/erythroid progenitor cells that are characteristic of CH were eliminated by rhG-CSF treatment in both CH dogs. These results suggest that the regulatory defect in canine CH can be temporarily alleviated by treatment with rhG-CSF and point to the potential treatment of human cyclic neutropenia with this agent.

CYCLIC HEMATOPOIESIS (CH), characterized by regular oscillations of the number of peripheral blood cells, is an inherited disease of gray collie dogs and some humans.1,2 In affected humans and dogs, neutrophils cycle more prominently than do other hematopoietic cells. Neutropenia recurs every 12 to 14 days in dogs and every 20 to 24 days in humans.3,5

In the CH dogs, the cycles of neutropenia are accompanied by almost concurrent cycles of thrombocytopenia and reticulocytosis, and they are followed by a marked monocytosis.4 Reticulocyte and thrombocyte cycles are much less distinct and more variable in humans than in dogs.3,4 Bone marrow cytology has shown that hematopoiesis occurs in waves in CH dogs and people.5,6,7 Hematopoietic progenitor cells, or colony-forming units (CFU), have also been shown to cycle in CH bone marrow cultures.8,9 The cycles of hematopoiesis are apparently not due to a shortened life span of circulating blood cells.10,11

Reciprocal bone marrow allografts between normal and CH dogs have shown that CH can be caused or induced by bone marrow transplantation.12,14 Similarly, human CH was induced coincidentally by transplantation of marrow from a CH patient to a leukemic sibling.15 Normal cellular life spans, alternating erythroid/erythroid bone marrow progenitor cells, cycles of hematopoietic cells of multiple lineages, and induction or cure of CH by marrow transplantation all strongly suggest that CH is a disease of the pluripotent stem cell.

Erythropoietin16 and colony-stimulating activity (CSA)17,18 have been shown to cycle inversely with cellular cycles in CH dogs. The erythropoietin cycles were observed in a normal dog after receiving a bone marrow allograft from a CH dog.19 Cycles of a macrophage migration inhibitory factor20 and a pre–B-cell growth factor have also been observed in CH dogs.21 The cycles of hematopoietic growth factors suggest that CH may result from a regulatory defect in the response to or production of hematopoietic growth factors. However, it is not known whether the cycles of hematopoietic growth factors are a cause or an effect of CH. The administration of endotoxin to CH dogs eliminated the characteristic hematopoietic cycles, presumably by causing a sustained production of CSA.22,23 Lithium therapy also was reported to eliminate the cycles of neutropenia, although these effects were not confirmed by another study.24,24

Recently, the major hematopoietic growth factors from both rodents and humans have been purified, and several have been molecularly cloned.25 Several different growth factors have been distinguished on the basis of the type of in vitro hematopoietic colony stimulated. The following growth factors, or colony stimulating-factors (CSF), have been identified: granulocyte CSF (G-CSF); granulocyte/macrophage-CSF (GM-CSF); monocyte-CSF (M-CSF); and multi-CSF, also known as interleukin-3.26,27 The in vivo activities of each CSF are compatible with the type of in vitro colony stimulated by that CSF.28,31 In normal animals, recombinant G and GM-CSF have been shown to cause an immediate and persistent leukocytosis.28,31 The leukocytosis was dependent on continued administration of a CSF, and the WBC count returned to normal within several days after stopping CSF therapy. In monkeys and mice, these factors reduce the neutropenia associated with myelosuppressive chemotherapy and/or lethal irradiation.28,31 Recombinant erythropoietin remedied the anemia of renal failure that is associated with human end-stage renal disease.24 However, in genetic disorders of hematopoietic proliferation, the effects of recombinant CSFs on hematopoiesis are not known. The purpose of
this study was to determine the effects of recombinant human G-CSF (rhG-CSF) and GM-CSF on hematopoiesis in normal and CH dogs.

MATERIALS AND METHODS

RhG-CSF and rhGM-CSF. The rhG-CSF expressed in Escherichia coli was purified as previously described.70 For administration to the dogs, the homogeneous rhG-CSF protein (0.5 mg/mL) in 10 mmol/L sodium acetate (pH 4.1) was diluted to a final concentration of 100 μg/mL with 25 mol/L HEPES-buffered phosphate-buffered saline (PBS) (pH 7.4) containing 1% heat-inactivated autologous dog serum. The rhG-CSF was administered subcutaneously (SC) at 10 μg/kg body weight divided twice daily. The rhGM-CSF was expressed in E coli and purified by sequential ion-exchange chromatography. The homogeneous GM-CSF protein (0.7 mg/mL), in 20 mmol/L Tris and 60 mmol/L sodium chloride (pH 7.5), was diluted to 100 μg/mL with HEPES-buffered PBS containing 1% heat-inactivated autologous dog serum. The rhGM-CSF was administered intravenously (IV) at 10 μg/kg body weight divided twice daily. The endotoxin contamination was <0.5 mg/mg protein for both rhG-CSF and rhGM-CSF as determined with the limulus amebocyte lysate assay.71 The specific activity of both rhG-CSF and rhGM-CSF was 1 × 1014 U/mg protein as determined with human bone marrow CFU-C assays of seven days for the rhG-CSF and 14 days for rhGM-CSF. One unit is defined as the amount of protein required to stimulate half-maximal colony formation in vitro.

Animals. The CH dogs used in this study were adult males between 1 and 2 years of age, in good health, and free of intercurrent infections. The colony of CH dogs has been described in detail previously.72 The normal controls were adult male mixed-breed dogs. CH dogs not undergoing treatment with CSF were used as untreated CH controls for marrow cell differential and marrow culture studies. All dogs were vaccinated for common infectious diseases, treated for parasites, and observed for 6 months before use in these studies. For collection of bone marrow, the animals were anesthetized with a combination of oxymorphone and xylazine. All dogs were housed in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Treatment protocol. The administration of rhG-CSF and rhGM-CSF to the CH dogs was begun on day 7 of the 14-day cycles. The first day of the cycle was designated as the first day the absolute neutrophil count decreased below 1,600/L. The cycles of the two CH dogs had been monitored for more than a year before the start of this study. The two CH dogs and one normal dog were treated with rhG-CSF for the first 30 days, observed for 30 days, and treated with the rhGM-CSF product during the last 30 days of the 90-day treatment period. Blood was obtained daily for serum analysis, WBC count including differential, platelet count, and reticulocyte count. Bone marrow from the treated dogs and the controls was collected into heparinized (50 U/mL) Iscove’s modified Dulbecco’s medium (IMDM) before the 90-day treatment period and twice weekly thereafter. Bone marrow smears were prepared and stained with Diff-Quik. Myeloid and erythroid progenitors were classified as previously described.73 Body temperatures were monitored daily.

CFU-GM. To determine the CFU-GM values, light-density nonadherent bone marrow cells (10⁸ to 10⁹/mL) were cultured at 37°C for seven days in IMDM supplemented with 15% fetal calf serum (KC Biologicals, Lenexa, KS) and 0.36% agarose. The rhG-CSF or rhGM-CSF were added at 1,000 U/mL cells, and CFU-GM were enumerated after seven days of culture. This is a modification of a procedure previously described in more detail.74

Detection of anti–G-CSF antibody. Serum levels of anti–G-CSF antibodies were determined indirectly by measuring the ability of each test serum to inhibit G-CSF–stimulated [³H] thymidine uptake in a cell line derived from a murine long-term bone marrow culture (NSF-60).75 A 1:100 dilution of dog serum was incubated with 10 units rhG-CSF for 30 minutes. The level of G-CSF remaining was then estimated by comparing the [³H] thymidine incorporation with the [³H] thymidine incorporation of known amounts of rhG-CSF not treated with dog serum.

RESULTS

rhG-CSF was well tolerated without apparent side effects and caused a rapid and persistent leukocytosis in both CH and normal dogs. The WBC count had increased to 20,000 by 12 hours after a single administration of 5 μg/kg G-CSF. In all three dogs the WBC count approached 60,000 after approximately 2 weeks of G-CSF treatment. The leukocytosis was due largely to a greater than tenfold increase in the number of circulating mature neutrophils (PMNs) in both the CH and normal dogs (Fig 1). A significant monocytosis was also observed in all three treated animals (Fig 1). Treatment with rhG-CSF blocked two predicted neutropenic episodes (as indicated by arrows) and established a persistent monocytosis in both CH dogs. The average cycle length before G-CSF administration in CH 356 was 13.9 days (n = 17 cycles) and 14.5 days (n = 18 cycles) in CH 357. However, during G-CSF treatment, neutropenia was absent for 33 days in CH 356 and for 34 days in CH 357, which was significantly (P < .0001) different, as determined by Student’s t test, from the pretreatment cycle length. The decline in PMNs and monocytes during the final five days of rhG-CSF treatment was accompanied by the development of neutralizing antibodies to rhG-CSF in all three dogs (Fig 2). The apparent antibody titer was indirectly determined by testing the ability of sera from the three treated dogs to block G-CSF–stimulated [³H] thymidine incorporation in murine NSF-60 cells.

Treatment with rhG-CSF appeared to eliminate the recurrent thrombocytosis in both CH dogs, but this could not be absolutely demonstrated since pretreatment platelet counts were not available. The rh-G-CSF treatment had no significant effect on the platelet values of the normal dog (data not shown). Pretreatment cycles of reticulocytes and eosinophils were not clearly recognizable in either of the CH dogs, and it was therefore not possible to determine whether rhG-CSF eliminated cycles of those blood cells. There was no obvious change in the number of circulating eosinophils or reticulocytes in any of the treated animals. The rhG-CSF caused an initial approximate doubling in the lymphocyte count in all three animals and eliminated the lymphocyte cycles in CH 356 (data not shown). Lymphocyte cycles were not apparent in CH 357 before or during rhG-CSF treatment.

rhGM-CSF administered to 2 CH dogs approximately 1 month after stopping the G-CSF treatment did not cause a significant leukocytosis or eliminate the cycles of neutropenia in either CH dog. A mild but significant (Student’s t test, P < .0005) increase in PMNs was noted in the normal dog during rhGM-CSF treatment. The mean ± SEM PMN count in the 14 days before rhGM-CSF treatment was 5,173 ± 225, which increased to 10,682 ± 442 during rhGM-CSF treatment. Although rhGM-CSF treatment caused a
significant increase in the number of PMNs, the absolute response was much less than that observed in the same animal during rhG-CSF treatment. Therefore, the dose and/or route of administration of rhGM-CSF was probably inadequate to evaluate its actions in the CH dogs.

The neutrophil cycle in peripheral blood is associated with marked changes in myeloid progenitor cells and in marrow morphology as indicated by myeloid/erythroid ratios (M/E). Late-stage neutrophils disappear in the marrow by day 7 of the cycle. Treatment with rhG-CSF prevented this marrow neutropenia, and the M/E ratio remained relatively constant throughout two cycles with G-CSF treatment.

In preliminary studies, maximum myeloid CFU growth in response to rhG- and rhGM-CSF was achieved at CSF concentrations of >500 U/mL (or 5 pg/mL) in normal and CH dog marrow cultures. Evaluation of bone marrow CFU-GM from normal and CH dogs treated with G-CSF showed an approximate doubling of marrow CFU-GM in the normal dog and elimination of the characteristic increase in marrow CFU-GM during the nadir of the neutrophil count in both CH dogs (Table 1). Bone marrow morphology and CFU-GM analysis in the CH dogs indicated that the elimination of neutrophil cycles with G-CSF treatment was associated with elimination of bone marrow progenitor cell cycles.

**DISCUSSION**

Purification and molecular cloning of the hematopoietic growth factors offers new potential for treating hematopoietic stem cell diseases. Since the hallmark of CH is recurrent cycles of neutropenia, we chose to test the effects of the granulocyte growth factors rhG-CSF and rhGM-CSF on hematopoiesis in CH dogs.

The administration of 5 μg/kg rhG-CSF twice daily to two CH dogs and one normal dog caused a persistent neutrophilia and monocytosis in all three dogs and prevented two predicted cycles of neutropenia in both CH dogs. The eventual decline in PMN and monocyte counts in the rhG-CSF treated dogs (Fig 1) was not due to a refractoriness or down regulation of the bone marrow stem cell pool since bone marrow cells generated a similar number of CFU-GM in response to rhG-CSF or rhGM-CSF (Table 1). Furthermore, the development of apparent neutralizing antibodies to
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day 7, midway between recurrent episodes of neutropenia. At episodes of neutropenia.4'

neutrophil demargination rather than to lation. However, Hammond et al treated a CH dog with 50

in the PMN count in the normal dog. The dogs and resulted in only a mild increase in the numbers of circulating PMNs and monocytes in the normal dog. The rhGM-CSF did not cause a significant change in the hematopoietic cycles in either of the CH dogs. The poor response of the dogs to the rhGM-CSF was probably not due to the presence of cross-reacting antibodies to rhGM-CSF from the previous rhG-CSF treatment since the apparent neutralizing antibodies to rhG-CSF declined during the rhGM-CSF treatment (Fig 2), but the poor response was most likely due to an ineffective dose and/or route of administration. Further, rhG-CSF and rhGM-CSF are unique proteins with little to no homology. Recently it has been shown that continuous IV infusions or SC injections of GM-CSF administered three times daily were necessary to cause a sustained leukocytosis in primates and humans.30,39,40 The mild increase in the PMN count in the normal dog could have been due to neutrophil demargination rather than to bone marrow stimulation. However, Hammond et al treated a CH dog with 50 µg/kg rhGM-CSF twice daily and found that the rhG-CSF treatment increased the peaks of neutrophilia during the recovery phase of the cycle but did not eliminate the episodes of neutropenia.41 Treatment with both growth factors was initiated on cycle day 7, midway between recurrent episodes of neutropenia. At this point in the cycle, in vitro growth is characteristically low, and we reasoned that a stimulus to the bone marrow at that stage in the cycle might prevent the subsequent episode of neutropenia. This proved to be the case with rhG-CSF treatment. This plan also provided an opportunity to study the effects of the growth factors on a cycle phase before the possible development of neutralizing antibodies.

Correction of the hematopoietic cycles in CH dogs with rhG-CSF suggests that rhG-CSF might be useful for treating human patients with cyclic neutropenia and possibly other types of myeloid aplasia. It is unlikely that the development of neutralizing antibodies, a problem in the CH dogs, would be a problem in humans since the protein is of human origin. Furthermore, resumption of the characteristic hematopoietic cycles in the CH dogs with the development of neutralizing antibody titers suggests that rhG-CSF therapy would require continual administration.

Acknowledgment

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References


Table 1. Bone Marrow CFU-GM in Normal and CH Dogs Treated With rhG-CSF

<table>
<thead>
<tr>
<th>Experimental Animal</th>
<th>CFU-GM (2.5 x 10⁶) Bone Marrow Cells</th>
<th>In Vivo Treatment</th>
<th>rhG-CSF</th>
<th>rhGM-CSF</th>
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</thead>
<tbody>
<tr>
<td>Normal dog</td>
<td>None</td>
<td>18 ± 1</td>
<td>27 ± 5</td>
<td></td>
</tr>
<tr>
<td>Normal dog</td>
<td>G-CSF</td>
<td>30 ± 4</td>
<td>60 ± 11</td>
<td></td>
</tr>
<tr>
<td>CH dog (nadir)</td>
<td>None</td>
<td>122 ± 10</td>
<td>390 ± 20</td>
<td></td>
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<tr>
<td>CH dog (recovery)</td>
<td>G-CSF</td>
<td>37 ± 4</td>
<td>57 ± 7</td>
<td></td>
</tr>
<tr>
<td>CH dog (nadir)</td>
<td>None</td>
<td>30 ± 4</td>
<td>57 ± 7</td>
<td></td>
</tr>
<tr>
<td>CH dog (recovery)</td>
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<td>27 ± 3</td>
<td>45 ± 5</td>
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<tr>
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<tr>
<td>CH dog (recovery)</td>
<td>G-CSF</td>
<td>28 ± 3</td>
<td>29 ± 3</td>
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</tr>
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</table>

Bone marrow was prepared and the CFU-GM assay performed as described by using either 1,000 units of rhG-CSF or 1,000 units of rhGM-CSF as a stimulus. Bone marrow responses in the two CH dogs were determined during neutrophil nadirs (cycle days 2 to 4) or neutrophil recovery (cycle days 6 to 8) before G-CSF treatment and at the time of a predicted nadir and predicted recovery period during G-CSF treatment.
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