

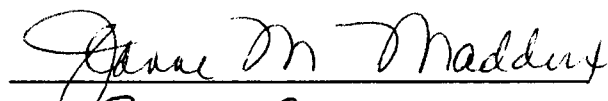
To the Graduate Council:

I am submitting herewith a thesis written by H. Lynn Pratt entitled "Signal Transduction Defects in Canine Cyclic Hematopoiesis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.



Clinton D. Lothrop Jr., Major Professor

We have read this thesis and
recommend its acceptance:




Accepted for the Council:



Vice Provost
and Dean of the Graduate School

**SIGNAL TRANSDUCTION DEFECTS
IN CANINE CYCLIC HEMATOPOIESIS**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

H. Lynn Pratt

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ABSTRACT

Canine Cyclic Hematopoiesis (CH) is an autosomal recessive disease of collie dogs, which is characterized by partial albinism and cyclic hematopoiesis at 14 day intervals. Previous studies on canine CH platelets have shown defective aggregation patterns to several agonists as well as a storage pool defects with decreased stores of dense granule nucleotides and serotonin. Recent studies have also identified defective phosphorylation of a 40kD protein which is the substrate for protein kinase C in platelets.

To further investigate the basis of phosphoinositide signal transduction defects in dogs with cyclic hematopoiesis, I compared platelet and neutrophil function in normal and CH dogs treated with recombinant human granulocyte colony stimulating factor (rhG-CSF). RhG-CSF eliminated neutrophil cycles associated with CH and eliminated endogenous G-CSF cycles in one CH dog and partially eliminated G-CSF cycles in another CH dog. However treatment with rhG-CSF failed to eliminate decreased platelet granule stores of serotonin or decreased neutrophil granule stores of myeloperoxidase (MPO). Moreover, defective thrombin, collagen and

platelet activating factor (PAF)-stimulated platelet aggregation and defective serotonin secretion persisted despite correction of the cell cycling in the CH dogs.

Superoxide production of CH dog neutrophils to three doses of phorbol myristate acetate (PMA) showed decreases relative to control dogs. Similar to the platelet storage pool defect, the MPO granule stores in CH dogs were also found to be decreased. However, CH neutrophils showed normal chemotaxis to three doses of PAF. In addition, when neutrophils were preincubated with cholera or pertussis toxin there were no significant differences in inhibition of PAF stimulated chemotaxis in the two groups of dogs.

The identification of similar defects in storage pools and signal transduction in platelets and neutrophils from CH dogs indicates a connection between storage pool function and the signal transduction cascade which may be involved in the pathophysiology of CH.

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CHAPTER I

INTRODUCTION

Canine cyclic hematopoiesis (CH) is an autosomal recessive disease of grey collie dogs.¹ The hallmarks of this disease are partial albinism and a cyclic neutropenia at 14 day intervals. Cells of other hematopoietic lineages also show asynchronous oscillation patterns.² Qualitative cellular defects of platelets and neutrophils are associated with CH.^{3,4} These defects are manifested clinically by an increased incidence of pyogenic infections and a mild bleeding tendency. Specific clinical signs in affected animals include fever, stomatitis, pneumonia, diarrhea, chronic abscesses, poor weight gain, chronic osteoarthritis and a tendency toward early death. Human cyclic neutropenia (CN) is a similar disease which is characterized by 21 day oscillations in neutrophil counts.⁵ This disease presents as both an inherited abnormality and as an adult onset condition.⁶ Although canine CH and human CN show many similarities, cyclic neutropenia has a milder clinical course.⁷

The etiology of CH has not been identified. The cycles of peripheral cells are not due to shortened

lifespans of circulating blood cells.⁸ Bone marrow cytology has shown an alternating erythroid-myeloid pattern that is compatible with the peripheral cell changes.⁹ CH cell cycles can be cured or induced by bone marrow transplantation, which implies a pluripotent stem cell defect.¹⁰

A large amount of research has focused on the qualitative platelet defects in this disease. Early work with CH dog platelets showed defective platelet adhesiveness and defective clot retraction that were independent of neutropenic episodes.¹¹ Defective platelet aggregation to collagen, platelet activating factor (PAF), thrombin and phorbol-12-myristate 13-acetate (PMA) are also present.³ Platelets from CH dogs have approximately 1/10 the amount of stored serotonin that is present in normal dog platelets. This is associated with a defect in the ability of CH platelets to take up and store serotonin.³ Adenine nucleotides, contained in the dense granules of platelets, are also decreased in CH dogs. Decreases of dense granule components classifies the disease among a group of heterogenous hereditary bleeding disorders known as storage pool disease.¹² Two of these disorders, Chediak-

Higashi syndrome and Hermansky-Pudlak syndrome also have associated pigment disorders.^{13,14}

Platelet dysfunction, characterized by the presence of storage pool disease and marked aggregation abnormalities to various agonists, appears to be as representative of CH as cell cycles and has persisted in the canine colony for 12 years despite multiple outcrossings. In addition, the presence of defective phosphorylation of a 40kD protein, the substrate for protein kinase C in platelets, has been identified.^{3,15} Platelet activation by agonists such as thrombin and collagen is mediated by the activation of membrane-bound phospholipase C which initiates the cleavage of phosphoinositols. This cleavage yields two important second messengers, 1,2-diacylglycerol(DAG) and inositol 1,4,5-triphosphate(IP₃).¹⁶ DAG activates protein kinase C, which subsequently phosphorylates a 40kD protein of unknown function in platelets.¹⁵ Defective phosphorylation of the 40kD protein suggests that CH dogs may have a biochemical defect in the phosphoinositide signal transduction pathway of platelet activation preceding 40 kD phosphorylation. The relationship between signal transduction and granule secretion is not clear. It has been clearly demonstrated that granule secretion

during aggregation causes an autocrine enhancement of the the aggregation response to agonists such as ADP and collagen. Storage pool disease is responsible in part for CH aggregation abnormalities but cannot be the sole cause of this defect since dogs heterozygous for the CH trait have normal stores of serotonin and ADP and yet have decreased collagen aggregation responses.³ Hermansky-Pudlak platelets, which are classified as storage pool deficient, have normal phosphorylation patterns but abnormal aggregation and secretion when thrombin-stimulated.¹⁷ Another storage pool disease, Chediak-Higashi syndrome has normal phosphorylation patterns to various agonists while having defective aggregation and secretion.¹⁸ CH platelets have normal phosphorylation and aggregation patterns when stimulated with high doses of thrombin. This points to a regulatory defect possibly in a feed-back loop of a signal transduction pathway. Since signal transduction pathways serve as common links between cells and their environment and mediate responses to a wide range of agonists such as hormones and growth factors, a regulatory defect in this pathway might explain diverse phenomena such as pigment dilution, waves of hematopoietic growth and cellular activation defects.

It is improbable that qualitative and quantitative cell defects of CH are caused by two different genetic defects. More likely, a single genetic aberration is responsible for both phenomena. A study by Dale et.al. showed qualitative neutrophil defects in CH dogs. They found CH neutrophils were capable of normal phagocytosis but had decreased intracellular killing of bacteria as well as decreased stores of myeloperoxidase.⁴ A complete characterization of cellular defects in neutrophils might elucidate the cause of platelet defects as well as the genetic lesion responsible for the disease. Neutropenia at regular intervals is the pathognomonic lesion of this disease, and yet only a minor amount of work with these cells has been done. If qualitative defects are present in CH neutrophils as the previous study suggests, then evaluation of biochemical pathways of neutrophil activation could conceivably ascertain the primary lesion of this disease.

Two bacterial toxins, pertussis and cholera, are often used as probes of neutrophil activation.^{19,20} Pertussis toxin is known to ADP ribosylate the alpha subunit of the guanine regulatory protein G_i . Adenylate Cyclase is controlled reciprocally by an inhibitory G protein (G_i) and a stimulatory G protein (G_s). Cholera

toxin ADP ribosylates the alpha subunit of G_s . These G proteins link the cell's receptor to the adenylate cyclase system and pertussis toxin is known to inhibit neutrophil activation by blocking G_i activation of the phosphoinositide pathway. The phosphoinositide signal transduction pathway is well characterized in neutrophils. It has been shown that certain agonists stimulate the breakdown of phosphatidylinositols which act in synergy with arachidonic acid release to stimulate neutrophil activation.²¹ Inhibition studies with pertussis and cholera toxin would provide information about this first step in the phosphoinositide pathway. Phorbol esters cause lysosomal enzyme secretion and superoxide production in neutrophils without increasing intracellular Ca^{2+} levels. Tumor promoters bind and directly stimulate protein kinase C, by-passing the first part of the pathway.²² It is also possible to measure intermediate products of the pathway such as phosphatidic acid or diacylglycerol or the translocation of protein kinase C from the membrane to the cytosol fraction upon activation. Since this pathway has been extensively studied in neutrophils, comparative studies with CH neutrophils should provide new information on neutrophil function in CH.

It is also conceivable that CH is a direct or indirect result of a defect in hematopoietic regulation. Erythropoietin, thrombopoietin and a colony stimulating factor (CSF) cycle in CH dogs.^{23,24,25} Cloning of many of the major hematopoietic growth factors has expanded our knowledge of hematopoiesis and the complex biological activities of these molecules.^{26,27} Growth factors have traditionally been distinguished on the basis of the type of in vitro hematopoietic colony stimulated. One such growth factor, G-CSF, is distinguished by its ability to stimulate primarily granulocytic colonies. When tested in vitro, recombinant human G-CSF (rhG-CSF) causes a leukocytosis in normal animals and reduces the neutropenia associated with myelosuppressive chemotherapy.²⁸ Administration of (rhG-CSF) to cyclic hematopoietic dogs caused a leukocytosis and eliminated the cellular cycles associated with this disease.²⁹ The leukocytosis was dependent upon continued administration of rhG-CSF and cell counts resumed cycling several days after stopping growth factor treatment. It has also recently been shown that G-CSF is produced cyclicly in CH dogs.³⁰ Peak amounts of G-CSF can be measured in serum immediately prior to the granulocyte recovery, implying a causative relationship between the two.

However, the effects of exogenous G-CSF on endogenous G-CSF cycles in CH dogs is not known.

Since rhG-CSF eliminated neutrophil cycles in CH dogs, we quantified platelet serotonin and platelet aggregation as well as neutrophil myeloperoxidase content before and during rhG-CSF treatment to determine if rhG-CSF corrected cell defects in CH platelets and neutrophils. In addition, G-CSF serum levels were measured prior to and during growth factor treatment to determine the effects of exogenous rhG-CSF administration on the endogenous cycles of G-CSF. One CH and one control dog were also treated by administration of pulsed doses of rhG-CSF for 3-4 days at various points during the CH cycle, to determine if continued administration of rhG-CSF was necessary to sustain the leukocytosis.

CHAPTER II

MATERIALS AND METHODS

Animals:

Adult CH dogs of both sexes, varying in age from 4 to 18 months were used in these studies. The colony has been previously described.³¹ Normal young adult dogs of both sexes were used as controls. All dogs were housed in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Neutrophil Isolation:

Neutrophils were isolated by differential centrifugation³² using Neutrophil Isolation Medium (Los Alamos Diagnostics, Los Alamos, NM). Whole blood (7mls) collected in EDTA tubes was layered onto 4 mls of neutrophil isolation media and spun at 400 X g for 30 minutes at room temperature. The neutrophil layer was aspirated and washed free of separation media using Medium 199 (Gibco, Grand Island, NY) with 1% fetal calf serum added. The contaminating red blood cells were lysed using lysing buffer (Los Alamos Diagnostics,, Los Alamos NM). Neutrophils were resuspended the final time in Medium 199. Total white cells were counted using a

particle counter (Coulter Electronics Inc., Hialeah, Fl). A differential cell count was performed on slides prepared in a cytospin centrifuge (Shandon, Pittsburgh, PA). Preparations consisting of 90-95% neutrophils were routinely obtained. Neutrophil viability using trypan blue exclusion was generally 95% or better.

Chemotaxis:

A 48 well micro chemotaxis chamber (Neuro Probe Inc., Cabin John, MD) was used in all experiments.³³ The apparatus was washed in pyrogen free water just prior to use. A 3uM polyvinylpyrrolidone-free polycarbonate membrane separated chemoattractants from cells.

Platelet activating factor (PAF) (Sigma, St. Louis, MO) was recieved as a chloroform solution (2mg/ml) and blown to dryness before resuspension in Medium 199 at 100, 10 and 1nm concentrations. Cholera and Pertussis Toxin (Calbiochem, LaJolla, CA and List Biological Labs, Campbell CA) were resuspended according to the manufacturer's instructions and diluted to 500 ng/ml in Medium 199.

The assembled apparatus was allowed to incubate at 37⁰C for 60 minutes. After incubation, the top plate, gasket and filter were removed, cells on the top were

wiped off and the filter was fixed in 8% glutaldehyde for 30 minutes. The cells were then stained for 2 minutes with Hucker's Crystal Violet.³⁴ The migrated cells were then counted with an image processor (Image Technology Corp., Deer Park, NY) connected to a light microscope.

Superoxide Production:

Superoxide was measured spectrophotometrically by the reduction of ferricytochrome C (Sigma, St. Louis, MO).³⁵ The continuous assay was performed using a Shimadzu UV-160 (Shimadzu, Kyoto, Japan) spectrophotometer equipped with a thermostatted cuvette holder. The cell suspension (5×10^6 /ml) in medium 199 was added to a 1 ml cuvette containing 60 μ m ferricytochrome c. The reference cuvette contained the above plus superoxide dismutase (Sigma, St. Louis, MO) at a final concentration of 62 μ g/ml. The cuvettes were warmed to 37°C before stimulation with phorbol myristate acetate (Sigma, St. Louis, MO). The change in absorbance at 550nm was followed and the millimolar amount of superoxide produced calculated from the linear part of the curve using 21.1 as the millimolar extinction coefficient for ferricytochrome c.

Myeloperoxidase:

Myeloperoxidase was measured in sonicates of isolated neutrophils. Neutrophils were isolated from peripheral blood as described.³²

Myeloperoxidase was measured by standard enzymatic methods.³⁵ Horseradish peroxidase was used to generate a standard curve and 4-aminoantipyrine was used as the substrate. The change in absorbance at 510 nm for 5 minutes was used to quantify the amount of myeloperoxidase present.

RhG-CSF Treatment Protocol:

Homogenous rhG-CSF protein (0.5 mg/ml) in sodium acetate pH 4.1 was diluted to a final concentration of 100 ug/ml with 25 mM Hepes in phosphate buffered saline containing 1% heat-inactivated autologous dog serum. This was administered subcutaneously at 10 ug/kg body weight, divided twice daily. Administration was begun on day 4 (CH 340) or 7 (CH 358) of the 14 day cycle for the cyclic dogs. CH 375 was treated with pulsed doses (3-5 days duration) of rhG-CSF to evaluate the ability of the growth factor to stimulate continuous myelopoiesis when begun on day 4 or day 7 during the cycle. A normal control dog was simultaneously treated. Blood was

obtained each morning for serum and plasma, and a white blood cell count, differential and platelet count was performed.

Platelet Aggregation:

Blood was collected by jugular venipuncture with 136 mM trisodium citrate as the anticoagulant, at a ratio of 1 part anticoagulant to 6 parts blood. Platelet-rich (PRP) and platelet poor-plasma (PPP) were prepared by differential centrifugation. PRP was directly applied to a Sepharose 2B Column (Pharmacia, Piscataway, NJ) at a ratio of 1:6, PRP: bed volume. Platelets were eluted from the column with modified Tangen-Hepes Buffer which consisted of 145mM NaCl, 15mM Hepes, 5mM KCl, 0.05mM CaCl₂, 0.1mM MgCl₂, 11mM glucose, 0.1% bovine serum albumin, pH 7.4.³⁶ After this procedure, the platelets retained their discoid shape and were unaggregated. The platelets were adjusted to a concentration of 3.0×10^8 platelets/ml using modified Tangen-Hepes buffer, and were evaluated in the experiments utilizing a dual channel aggregometer (Payton Associates Inc. Buffalo, NY). A 50 ul aliquot of a 10-fold concentrated agonist was used to induce aggregation. The extent of aggregation was calculated from the maximal change in light transmission.

The slope of the aggregation tracing was used to approximate the initial velocity of aggregation.

Serotonin Quantification:

Platelet serotonin content was determined for all the animals in two separate experiments both before and after rhG-CSF treatment. Serotonin was determined by the original method of Crosti and Lucehelli as modified by Holmsen and Weiss.³⁷ Standard curves were generated by using known amounts of serotonin creatinine sulfate (Sigma, St. Louis, MO). Fluorescence was determined using a 650S fluorescence detector (Perkin Elmer Corp, Norwalk, Conn) interfaced with a Fischer Recordall Series 5000 chart recorder (Fischer, Pittsburg, PA) with an excitation wavelength of 295nm and an emission of 540nm.

Serotonin Secretion:

Platelet rich plasma was incubated with [¹⁴C]-serotonin (Amersham, Arlington Heights, Ill) at 0.05uCi/ml for 30 minutes at 37°C. The platelets were gel-filtered to remove unincorporated isotope. Gel-filtered platelets were diluted to 3×10^8 platelets/ml with the Tangen-Hepes buffer. Platelets were warmed to 37°C and then stimulated with collagen or thrombin. A 100 ul sample aliquot was added to 900 ul of ice-cold PBS

containing 0.1% glutaldehyde and the platelets were pelleted by centrifugation. The radioactivity in 0.5 mls of the supernatant was used to estimate percent serotonin secretion. Total secretable serotonin was determined on an equal aliquot of platelets which had not been stimulated with an agonist or concentrated by centrifugation.

NFS-60 Proliferation Assay:

G-CSF biologic activity was measured by Maryanne Maniatis and David Warren, Memorial Sloan-Kettering Cancer Center, NY, in a standard cell proliferation microassay using the NFS-60 cell line which is derived from murine long term bone marrow culture.³⁸ NFS-60 cells (10^4 /well) were cultured in RPMI 1640 containing 2% fetal calf serum in flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA) containing varying concentrations of test samples. After 18 hours incubation at 37°C in a fully humidified atmosphere of 5% CO₂ in air, cultures were pulsed for 4 hours with 0.5 uCi methyl[³H]-thymidine (specific activity, 20 Ci/mmol; New England Nuclear, Boston, MA). Cells were harvested on an automatic cell harvester, and ³H-TdR incorporation measured by liquid scintillation counting.

Assay results are expressed as a mean of triplicate cultures.

Statistical Methods:

All data is presented as mean $\pm$ SEM. All n numbers refer to the number of individual animals tested. Statistical significance was determined using Student's t test.

CHAPTER III

RESULTS

Chemotaxis:

Chemotaxis was measured using 1, 10 and 100 nanomolar doses of PAF as the chemoattractant. (Table 1)* With 100 nM PAF, CH neutrophils had a 5.1 ± 3.8 fold-increase relative to background migration while control dogs had a 5.1 ± 2.7 fold-increase. At 10 nM, CH neutrophils had a 4.0 ± 2.0 fold increase while neutrophils from normal dogs showed 3.7 ± 1.4 fold-increase. With 1 nM PAF CH neutrophils had a 4.0 ± 1.5 and normal dog neutrophils showed 3.5 ± 2.2 fold-increase. There were wide ranges of daily responses of both normal and CH neutrophils. However there was no significant difference in the response of CH dog neutrophils compared to normal dog neutrophils at any of the doses of PAF tested. This contrasts with CH dog platelets which have defective aggregation patterns with PAF as the stimulant. Neutrophils also showed chemoattractant responses to normal dog serum and zymosan activated serum (data not shown).

*All tables and figures can be found in the appendices.

Both pertussis and cholera toxin inhibited neutrophil chemotaxis to PAF. (Table 2) Cholera toxin (500 ng/ml) inhibited chemotaxis of CH dog neutrophils to 100 nM PAF by 68.8 ± 10.5 percent. Control dog neutrophils were inhibited by 62.0 ± 11.7 percent. Pertussis toxin (500ng/ml) was a more effective inhibitor, causing 83.0 ± 10.9 percent inhibition of chemotaxis in control dog neutrophils and 77.0 ± 15 in CH dog neutrophils. Neutrophils of control animals showed a linear dose response to pertussis toxin inhibition which was not evident in CH dogs. (table 2)

Superoxide Production:

Superoxide production of CH and normal dog neutrophils was measured after stimulation with 10, 1 and 0.1 ug/ml doses of PMA. (Table 3) With 10 ug/ml of PMA, control dogs produced 0.81 ± 0.12 nmoles of superoxide/minute/ 10^6 cell, while CH dogs produced 0.44 ± 0.11 . With 1 ug/ml PMA control dogs produced 0.89 ± 0.19 while CH dogs produced 0.60 ± 0.24 . At 0.1 ug/ml PMA control dogs produced 0.75 ± 0.23 while CH dogs produced 0.54 ± 0.16 . The total amount of superoxide produced by dog neutrophils was slightly decreased from that reported for human neutrophils stimulated with

PMA.³⁹ Overall CH neutrophils produced less superoxide than normal dogs, particularly with 10 ug/ml, although the difference was not statistically significant.

Effect of RhG-CSF on Neutrophil and Platelet Cycles in CH Dogs:

Administration of rhG-CSF was well tolerated without apparent side effects. The WBC counts of all the dogs increased to 25,000/ ul within 24 hours after treatment was initiated. This was due to a marked monocytosis and neutrophilia. Treatment with rhG-CSF blocked predicted neutropenic episodes in two of the treated animals as previously shown.²⁹ The decline in cell counts after 15 days of treatment was most likely due to the development of neutralizing antibodies to rhG-CSF.²⁸

CH 340 was treated with rhG-CSF 10 ug/kg body weight for 35 consecutive days. (Fig. 1) Her counts remained increased for 15 days. Following this, the dog became persistently neutropenic until her death 7 days after treatment was discontinued.

CH 358 was treated with rhG-CSF 10 ug/kg body weight for 25 consecutive days. (Fig. 2) After 15 days of treatment the animal's WBC counts returned to normal

levels for 3 days, then escalated tremendously. After ceasing treatment, the animal's neutrophil cycles resumed.

CH 375 was treated with rhG-CSF 10 ug/kg of body weight for 3 consecutive days beginning on cycle day 4. (Fig. 3) This caused an increase of the WBC count to 35,000/ul but not until 72 hours after treatment was begun. The cell counts remained increased for 48 hours before returning to normal levels. The animal was treated for 3 days again at the predicted neutrophil nadir. A peak neutrophil count of 20,000/ul was reached and sustained for 24 hours. The neutrophils then resumed their normal cycle. Treatment was continued at cycle day 6 of the next cycle and continued for 6 consecutive days (until cycle day 12). This caused an increase of the total WBC count to 40,000/ul and a neutrophil nadir of 3600/ul on cycle day 2 for that cycle. The animal's neutrophil resumed cycling after cessation of treatment.

One normal dog was treated with rhG-CSF for 27 consecutive days. (Fig 4) This animal's WBC count rose to 25,000/ul within 24 hours of the initiation of treatment and were sustained at 50,000 one week into treatment. WBC counts were sustained for 20 days. After

this time the animal became neutropenic and remained so for 8 days following the cessation of treatment, at which time the animal was euthanised.

The other control animal was treated with pulsed doses of rhG-CSF concurrently with CH 375. (Fig. 5) The WBC counts rose to 50,000/ul during treatment but quickly dropped when the growth factor treatment was stopped. This animal did not become persistently neutropenic.

Platelet counts were also determined prior to and during rhG-CSF treatment. Pretreatment platelet cycles were noticeable only in CH 358. (Fig. 6) The typical thrombocytosis was present in the three CH dogs. (not shown) There was no obvious effect of rhG-CSF on platelet counts except for a sustained thrombocytosis in CH 358 during treatment.

Effect of RhG-CSF on Endogenous G-CSF Cycles in CH Dogs:

G-CSF biologic activity was measured in the serum of a CH and a normal dog before and during treatment with rhG-CSF by a NFS-60 proliferation assay. Two expected peaks of G-CSF were eliminated by growth factor treatment in CH 340 (Fig. 7). CH 358 showed two peaks of G-CSF during rhG-CSF treatment that were shifted relative to their expected occurrence and decreased in

size. (Fig. 8) The normal dog experienced a slight rise in background counts during treatment but did not have a cyclic pattern of endogenous G-CSF. (Fig. 9) CH 375, treated with pulsed doses of rhG-CSF showed the expected peaks of G-CSF. (Fig. 10)

Platelet Aggregation:

After stimulation with collagen, thrombin or platelet activating factor (PAF), platelets from CH dogs differed significantly in both the extent and the rate of platelet aggregation, both before and during growth factor treatment. (Table 4) At low doses of two agonists (collagen 5 ug/ml and PAF .001 uM) platelets from CH dogs showed only half the extent of aggregation that was seen in the control dogs. The CH dogs had a stronger response to low doses of the agonist thrombin (.01 U/ml) but still had a decreased response relative to the control dogs. This did not change when aggregations were performed 12 days after beginning growth factor treatment for the 2 CH and 1 normal dog (approximately 4 platelet maturation times).

Serotonin Quantification:

CH dog platelets had approximately 10% the serotonin content of normal dog platelets. This has been well

documented.³ Serotonin content (ng/10⁹ platelets) of CH platelets (n=9, 6.6±.9) was markedly reduced relative to the normal dog platelets (n=6, 135.8±9.0). Serotonin content was slightly increased during growth factor treatment in CH dogs (n=6, 13.0±1.7) and decreased in normal dogs during growth factor treatment (n=4, 94.0±11.7).

Serotonin Secretion:

Platelets from CH dogs were unable to take up and retain [¹⁴C]-serotonin as evidenced by the increased radioactivity in the supernatants of unstimulated samples relative to normal dog platelets. (Table 5) There was no further release of [¹⁴C]-serotonin from CH platelets after agonist stimulation. Platelets from normal dogs retained [¹⁴C]-serotonin and then secreted more than 65% of the [¹⁴C]-serotonin pool after collagen stimulation and more than 95% after thrombin stimulation. There was no improvement in [¹⁴C]-serotonin retention or secretion in platelets from a CH dog treated with rhG-CSF for 12 days.

Myeloperoxidase:

Myeloperoxidase is a heme enzyme present in the primary granules of neutrophils. It is decreased by

approximately half in neutrophils from CH dogs when compared to normal dogs.⁴ The neutrophils of a large group of CH dogs (n=12), which included those used in these studies, had approximately 20% the amount of myeloperoxidase of normal dogs neutrophils (n=12). (CH dogs 0.43 ± 0.13 , normal dogs 2.72 ± 0.88) During rhG-CSF treatment both CH (n=2) and control dogs (n=2), (with two determinations on each dog) had increases in MPO content but the CH dogs remained deficient compared to normal controls (CH dogs 1.13 ± 0.05 , control dogs 4.06 ± 1.35).

CHAPTER IV

DISCUSSION

Neutrophil function of CH dogs has not been completely evaluated. Early work showed a decrease in the amount of myeloperoxidase in CH dog neutrophils as well as a decrease in their bactericidal activity.⁴ Since this work was done, there has been an expansion in our understanding of the biochemical pathways responsible for neutrophil activation. If qualitative defects of activation are present in CH neutrophils, then a biochemical evaluation of those defects could conceivably ascertain the cause of platelet defects, as well as cell cycles and the primary lesion of the disease.

It has been clearly demonstrated that many receptors are coupled to effector molecules by G proteins in the membrane. The role of G proteins in regulating adenylate cyclase has been well characterized.⁴⁰ G protein(s) have also been shown to raise intracellular Ca^{+2} levels thereby activating membrane bound phospholipase C to cause the hydrolysis of phosphoinositides (PI). Two important second messengers result from the breakdown of phosphoinositides: 1,2-diacylglycerol and inositol 1,4,5-triphosphate. Diacylglycerol activates protein kinase C

(PKC) while inositol 1,4,5 triphosphate (IP_3) causes the mobilization of intracellular calcium pools.⁴¹ Defects in activation of platelets from CH dogs led to an evaluation of this second messenger pathway. Phosphorylation of a 40kD protein by protein kinase C was found to be decreased in CH platelets.⁴ Evaluation of PI hydrolysis in another cell lineage is important to understanding the pathophysiology of CH. Phosphoinositide metabolism has been shown to occur in PMN's exposed to PAF, LTB_4 , and C5a.⁴² PAF, has been shown to stimulate protein kinase C activity in human neutrophils as well as stimulating superoxide anion production, aggregation, adherence and chemotaxis. PMA can directly stimulate protein kinase C in isolated cytosolic fractions.⁴³ Pertussis and cholera toxin ADP ribosylate the alpha subunit of G_i and G_s , respectively causing an alteration in activity of the G protein which results in an activation of the adenylate cyclase system.⁴² Extension of platelet work to the granulocytic lineage was deemed appropriate to further investigate signal transduction in CH.

Our studies support earlier work by identifying decreased stores of MPO in CH granulocytic lineage. Myeloperoxidase, a heme enzyme stored in the primary granules of neutrophils, functions in the termination of

the respiratory burst.⁴⁴ MPO granule enzyme deficiency is often seen in myeloproliferative disorders and can be related to the functional maturity of the granulocyte.⁴⁵ It is possible that CH as a disease results in general dysfunction of granules, since there is also a decrease in platelet dense granule contents.

Chemotaxis of normal and CH PMNs to 100.0, 10.0, and 1.0 nM doses of PAF was found to be similar. PAF has been shown to stimulate PKC activity in the particulate fraction of human neutrophils. It has also been found to stimulate phosphoinositide metabolism in platelets and PMNs. Normal PAF induced chemotaxis and suggests normal PI signal transduction. It is possible that there exists a threshold of CH PMN response to PAF induced chemotaxis, much like the CH platelet responses, that our studies did not reveal. Without observations at lower doses of PAF it is impossible to rule out signal transduction abnormalities.

Both pertussis and cholera toxin inhibited PAF induced chemotaxis. The fact that PT inhibits PAF-mediated effects on neutrophils is suggestive of the involvement of a G_i like protein. Apparently there is a G protein acting as a pertussis toxin substrate that is immunochemically distinct from G_i .^{42,46} PT causes a

NAD-dependent ADP ribosylation of α_i G protein subunit which blocks its dissociation from β and blocks the inhibitory action mediated by G_i . Pertussis toxin causes no measurable increase in cAMP levels, which suggests that the G protein affected by pertussis toxin in neutrophils is not coupled to adenylate cyclase.⁴⁶ Pertussis toxin has been shown to inhibit Ca^{+2} mobilization induced by fMLP, which is mediated by IP_3 . Neutrophils show a similar to normal PT inhibition pattern when stimulated by PAF. This provides information about only one part of an early event in PI signal transduction and does not evaluate the rest of the pathway. Also the fact that G proteins in CH PMN's respond normally to PT inhibition does not imply that the entire protein is normal. Cholera toxin causes an ADP ribosylation of α_s which blocks GTPase action and causes adenylate cyclase to be persistently activated. Cholera toxin has been shown to cause an elevation in cAMP levels in PMNs which is known to inhibit chemotaxis. Normal CT inhibition of PAF induced chemotaxis in CH PMN's implies a normal adenylate cyclase system and cellular responses to cAMP.

Superoxide production of CH neutrophils to 10.0, 1.0 and 0.1 μ g/ml PMA was found to be decreased at all doses compared to normal dogs, but was only significant

($P < 0.05$) at the high dose. PMA stimulates PKC activity in isolated cytosolic fractions of PMNs. It is a strong stimuli of superoxide anion production and degranulation and is not sensitive to PT inhibition. PMN responsiveness to PMA is primarily mediated by PKC activation with little if any requirement for IP_3 mediated Ca^{+2} increase, unlike receptor coupled PMN activation which is synergized by Ca^{+2} flux.⁴⁷ The decreased PMA-induced superoxide anion production in CH PMN's is consistent with a decreased PMA-induced aggregation pattern seen in CH platelets. This could imply abnormal activity of PKC in CH disease or abnormalities in phosphorylation of proteins stimulated by PKC. It is also possible that a PKC inhibitor is present. Some substances known to inhibit PKC activity are calmodulin, troponin C, cAMP, calpain, sphingosine and lysosphingolipids. It has recently been reported that there is a rapid down-regulation of PKC in the beige mouse which is an animal model for Chediak-Higashi syndrome, a disease that possesses many similarities to CH.⁴⁸ PKC in CH disease warrants further biochemical investigation, such as measuring the translocation of PKC and the phosphorylation of proteins after PKC stimulation.

The growth factor, G-CSF, is one member of a family of hematopoietic growth factors that is required for proliferation and differentiation of granulocyte precursors. It stimulates granulopoiesis in vivo and accelerates recovery from myelosuppressive chemotherapy.²⁸ Mature granulocytes retain receptors for G-CSF, and, when tested in vitro, this protein has been shown to prime cells and stimulate such functions as superoxide production and phagocytosis.⁴⁹ Since qualitative cellular defects in canine CH have been documented, we wanted to investigate the in vivo effects of this growth factor on mature hematopoietic cell function as well as cell cycles. Secondly, since it has recently been shown that there are regular oscillations in serum G-CSF levels in canine CH,³⁰ we wanted to investigate the effect of administration of exogenous rhG-CSF on these naturally occurring G-CSF cycles. Thirdly, we investigated the feasibility of administration of pulsed doses of rhG-CSF to eliminate the CH cycle. The fact that this growth factor has been shown to alleviate the regulatory defect of hematopoiesis in canine CH, points to an important but unclear role for this growth factor in this disease.

Administration of 5 ug/kg rhG-CSF twice daily, a superphysiologic dose, to two CH dogs and one control dog

caused a neutrophilia and monocytosis in all three dogs, and prevented a cycle of neutropenia in the CH dogs. The eventual decline in cell counts was most likely due to the development of neutralizing antibodies in the animals on continuous treatment, as previously shown.²⁹ Two animals became persistently neutropenic after continuous rhG-CSF treatment, suggesting a cross reactivity of the human product with dog G-CSF that prevents neutrophil production. The experimental and control animals treated with pulsed doses of rhG-CSF increased WBC counts during treatment that returned to normal shortly after treatment was stopped. The pulsed CH dogs' treatment was initiated on day 4 and day 7 respectively, of a 14 day cycle, which is defined by day 1 being the day the neutrophil nadir is less than 1500 PMN/ul. Pulsed treatment initiated on day 7, the point in the cycle when in vitro hematopoietic cell growth is characteristically low, resulted in a delayed neutrophil nadir for the next cycle. Apparently the cycle was reset but not blocked. Treatment initiated on day 4 did not affect the cycle. The pulsed control animal's WBC counts returned to normal within 48 hours of cessation of treatment.

Evaluation of aggregation patterns to three agonists before treatment showed a significant decrease in the response of the CH platelets relative to the

normal dog platelets. Evaluation of platelet aggregations of all five treated dogs, when cell counts were markedly elevated, showed no change in defective CH aggregation patterns. Platelet serotonin content was also evaluated before and during treatment for the animals under study. There was no significant improvement in the serotonin content of treated CH animals platelets. RhG-CSF has been shown to shorten thrombocytopenia associated with myelosuppression, although it is not known it's direct or indirect effect on platelets.²⁸ The fact that neutrophil counts in two of the CH dogs on the study had been increased for two weeks, or 3-4 platelet generation times,⁵⁰ supports the theory that CH platelet defects are not secondarily caused by neutrophil cycles. This observation is important to the study of CH platelet defects as well as the study of the role of G-CSF in this disease.

We also evaluated rhG-CSF's effect on decreased levels of MPO of the granulocytic lineage of CH. As in the platelets, no change was seen in the defect before and during treatment. Decreased stores of MPO in the granulocytic lineage of two CH dogs were not corrected by the administration of rhG-CSF. The cellular defects in platelets and neutrophils associated with CH appear to be primary to the disease, rather than secondarily caused by

the cell cycles. MPO deficiency is a common finding in myeloproliferative disorders, with the highest incidences in cases with the greatest differentiation potential. The deficiency tends to correlate with prognosis since it tends to disappear during remission and reappear during relapse.⁴¹ Administration of rhG-CSF to CH dogs did not correct the CH MPO deficiency despite the fact that there was a great increase in circulating neutrophils. The stem cell(s) stimulated by rhG-CSF carried the CH defect. This is an important consideration when canine CH or human CN is treated with rhG-CSF.

It has recently been shown that G-CSF shows a cycling pattern in sera of CH dogs. It appears that this is the cause of the neutrophil recovery that occurs after the neutrophil nadir, since peaks of G-CSF always precede the peripheral neutrophilia.²⁸ It is unknown though, if cyclic G-CSF production is a cause or an effect of the neutropenia. Since G-CSF has been shown to have a very short half life in serum in human trials, we measured endogenous G-CSF in daily serum samples collected 12 hours after the previous treatment with rhG-CSF. Treatment with rhG-CSF eliminated two of one of the CH dog's endogenous peaks of G-CSF. The control dog showed only a slight increase in background counts during G-CSF treatment. This shows a clear relationship

between neutrophil counts and levels of G-CSF in this disease. High levels of G-CSF maintain high cell counts which then shut off G-CSF production. G-CSF is produced in macrophages, monocytes and endothelial and fibroblast cells of the bone marrow stroma. During the immune response, IL-1 stimulates monocytes which in turn produce G-CSF and cause a priming and proliferation of granulocytes. IL-1 is also able to prime early progenitor cells to respond to M-CSF and GM-CSF. Studies such as this indicate that the CH dog is a unique opportunity to explore the in vivo regulation of hematopoietic growth factor production.

The above studies showed that pulsed doses of G-CSF were not sufficient to eliminate CH neutrophil cycles, cell defects were not corrected by rhG-CSF treatment and endogenous cycles of G-CSF were eliminated by exogenous rhG-CSF treatment in one CH dog. It is evident that CH cells are capable of responding to G-CSF. Certainly the production of G-CSF is not normally controlled in CH dogs. The disease affects cells outside of the hematopoietic lineage and affects the hematopoietic lineage in more ways than just growth and proliferation. The fact that defects in cell function remain during tremendous cell proliferation points to a primary stem cell defect.

The control of normal hematopoiesis is believed to operate with undetectable intrinsic rhythms that border on oscillations. Recently, treatment of a patient affected with idiopathic neutropenia with rhG-CSF resulted in increased WBC cell counts and natural 40 day oscillations of monocytes, neutrophils and platelets.⁵¹ Clearly, CH as well as other diseases of defective hematopoietic regulation, are a result of an abnormality in dynamic responses possibly involving other growth factors as well as committed and uncommitted stem cell pools. It is hopeful that certain genetic defects in the control of hematopoiesis, such as CH will lead to our unraveling the mysteries and complexities of hematopoietic stem cell regulation.

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APPENDICES

APPENDIX A

TABLE 1
CHEMOTAXIS OF NORMAL AND CYCLIC
HEMATOPOIETIC DOG NEUTROPHILS TO
PLATELET ACTIVATING FACTOR

PAF (nM)	N	CH
100.0	5.1 $\pm$ 2.7 (n=12)	5.1 $\pm$ 3.8 (n=12)
10.0	3.7 $\pm$ 1.4 (n=7)	4.0 $\pm$ 2.0 (n=7)
1.0	3.5 $\pm$ 2.2 (n=6)	4.0 $\pm$ 1.5 (n=5)

Data is expressed as relative increases over background migration. Data is summarized as mean $\pm$ SEM. There are no statistically significant differences.

TABLE 2

CHOLERA AND PERTUSSIS TOXIN
INHIBITION OF CHEMOTAXIS STIMULATED BY
0.1 μ M PLATELET ACTIVATING FACTOR

TOXINS ng/ml	N	CH
PERTUSSIS		
500.0	83.0 $\pm$ 10.9 n=3	77.0 $\pm$ 15.0 n=3
250.0	71.5 $\pm$ 11.9 n=5	43.8 $\pm$ 11.3 n=3
125.0	59.5 $\pm$ 19.0 n=5	73.8 $\pm$ 13.6 n=3
66.0	48.3 $\pm$ 21.1 n=4	67.7 $\pm$ 15.4 n=4
CHOLERA		
500.0	62.0 $\pm$ 11.7 n=6	68.8 $\pm$ 10.5 n=7

Data is expressed as % inhibition of PAF stimulated cells and expressed as mean $\pm$ SEM. There are no statistically significant differences.

TABLE 3
 SUPEROXIDE PRODUCTION OF NORMAL
 AND CYCLIC HEMATOPOIETIC DOG NEUTROPHILS

Phorbol Myristate Acetate PMA (ug/ml)	N	CH
10.0	.81 $\pm$.12 (n=4)	.44 $\pm$.11 (n=5)
1.0	.89 $\pm$.19 (n=5)	.60 $\pm$.24 (n=5)
0.10	.75 $\pm$.23 (n=3)	.54 $\pm$.16 (n=4)

Data is expressed as nmoles/O₂/min 10⁶ cell and summarized as mean $\pm$ SEM. There are no statistically significant differences.

TABLE 4
PLATELET AGGREGATION IN NORMAL AND
CYCLIC HEMATOPOIETIC DOGS BEFORE AND DURING G-CSF TREATMENT

AGONIST	CH			N	
	PRE (n=3 dogs)	TREATED (n=2)	PRE (n=2)	TREATED (n=1)	
Collagen	20.0 ug/ml	30.6 $\pm$ 12.9	30.6 $\pm$ 5.9	59.7 $\pm$ 4.9	69.6 $\pm$ 11.3
	5.0 ug/ml	6.0 $\pm$ 4.0	9.2 $\pm$ 4.5	59.0 $\pm$ 4.4	66.1 $\pm$ 6.2
Thrombin	1.00 U/ml	58.8 $\pm$ 18.8	48.2 $\pm$ 6.5	76.1 $\pm$ 8.3	79.4 $\pm$ 8.3
	0.01 U/ml	42.9 $\pm$ 2.8	48.2 $\pm$ 6.5	60.3 $\pm$ 1.2	67.1 $\pm$ 6.4
PAF	0.01 uM	52.3 $\pm$ 6.4	29.2 $\pm$ 5.4	60.8 $\pm$.15	79.4 $\pm$.25
	0.001 uM	27.5 $\pm$ 10.5	30.9 $\pm$ 13.5	50.1 $\pm$ 3.7	51.1 $\pm$ 1.1

The data is summarized as percent aggregation and expressed as mean $\pm$ SEM. N - number indicates individual animals. There were two observations on each animal.

TABLE 5
 SEROTONIN SECRETION BY PLATELETS FROM NORMAL
 AND CH DOGS BEFORE AND DURING G-CSF TREATMENT

TIME (MINUTES)	PRETREATMENT				G-CSF TREATED			
	COLLAGEN		THROMBIN		COLLAGEN		THROMBIN	
	NORMAL (n=3)	CH (n=3)	N (n=3)	CH (n=3)	NORMAL (n=1)	CH (n=1)	NORMAL (n=1)	CH (n=1)
0	5.9±2.3	67.0±14.1	3.7±3.5	75.5±19.1	3.7	44.5	4.8	54.2
2	33.7±32	55.3±7.3	96.5±6.1	78.8±17.7	11.8	42.6	94.1	69.3
4	60.5±14.9	60.4±8.4	97.3±4.7	74.9±11.9	62.7	45.9	95.6	69.6
6	66.6±12.5	58.8±9.0	97.8±3.1	78.2±13.0	65.3	47.4	96.4	70.1
8	64.1±5.7	65.9±22.8	95.7±5.0	79.7±13.0	43.6	48.6	97.4	62.6

The data is summarized as mean ± SEM. Serotonin secretion was quantitated as described on 100 ul aliquots of platelets immediately prior to and 2, 4, 6 and 8 minutes after collagen (20 ug/ml) or thrombin (0.01 U/ml) stimulation.

APPENDIX B

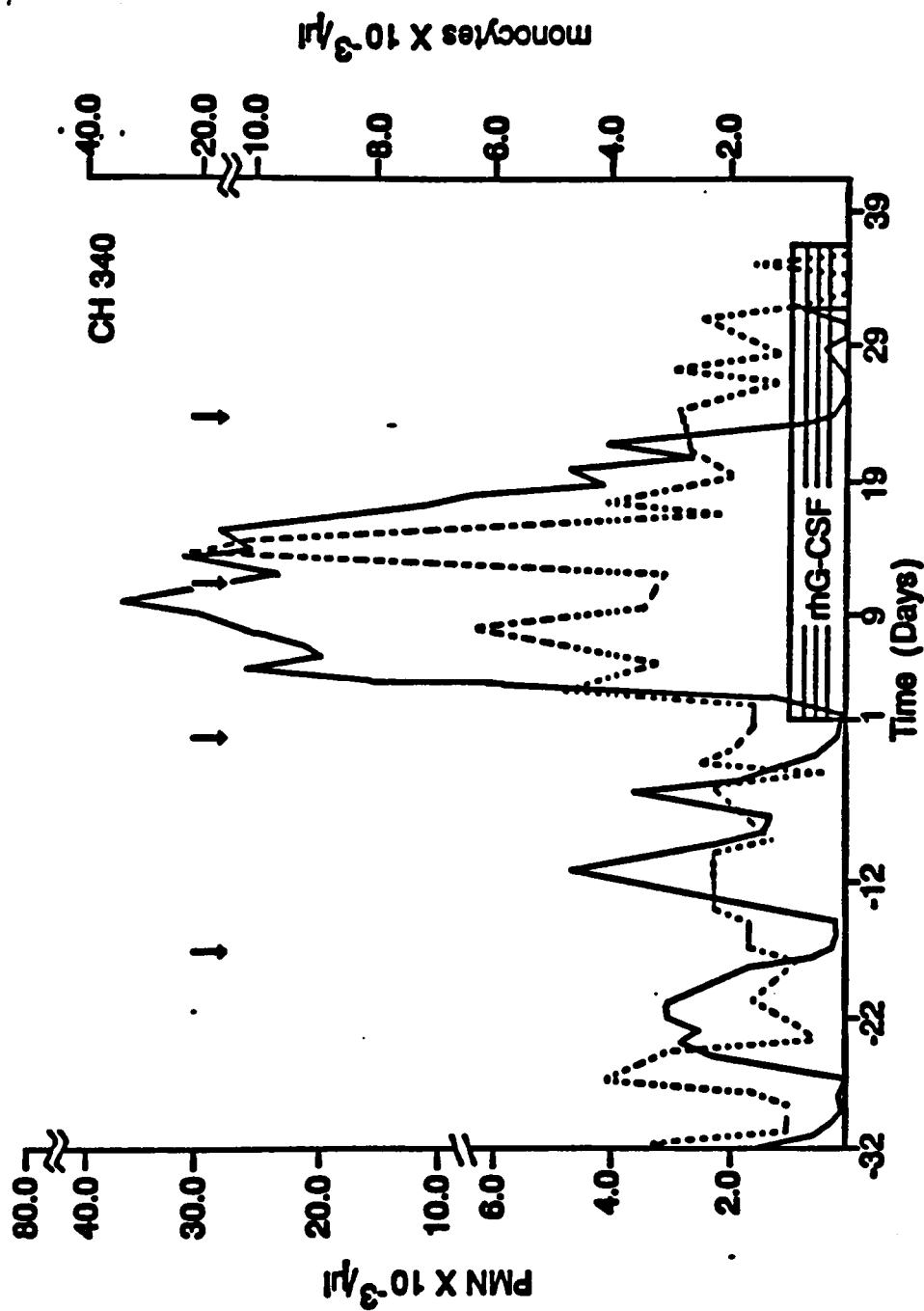


Figure 1. The effect of rhG-CSF on PMN counts (solid line) and monocyte counts (dashed line) in CH 340. Arrows indicate times of predicted neutropenia. Day 1 is the first day of rhG-CSF treatment.

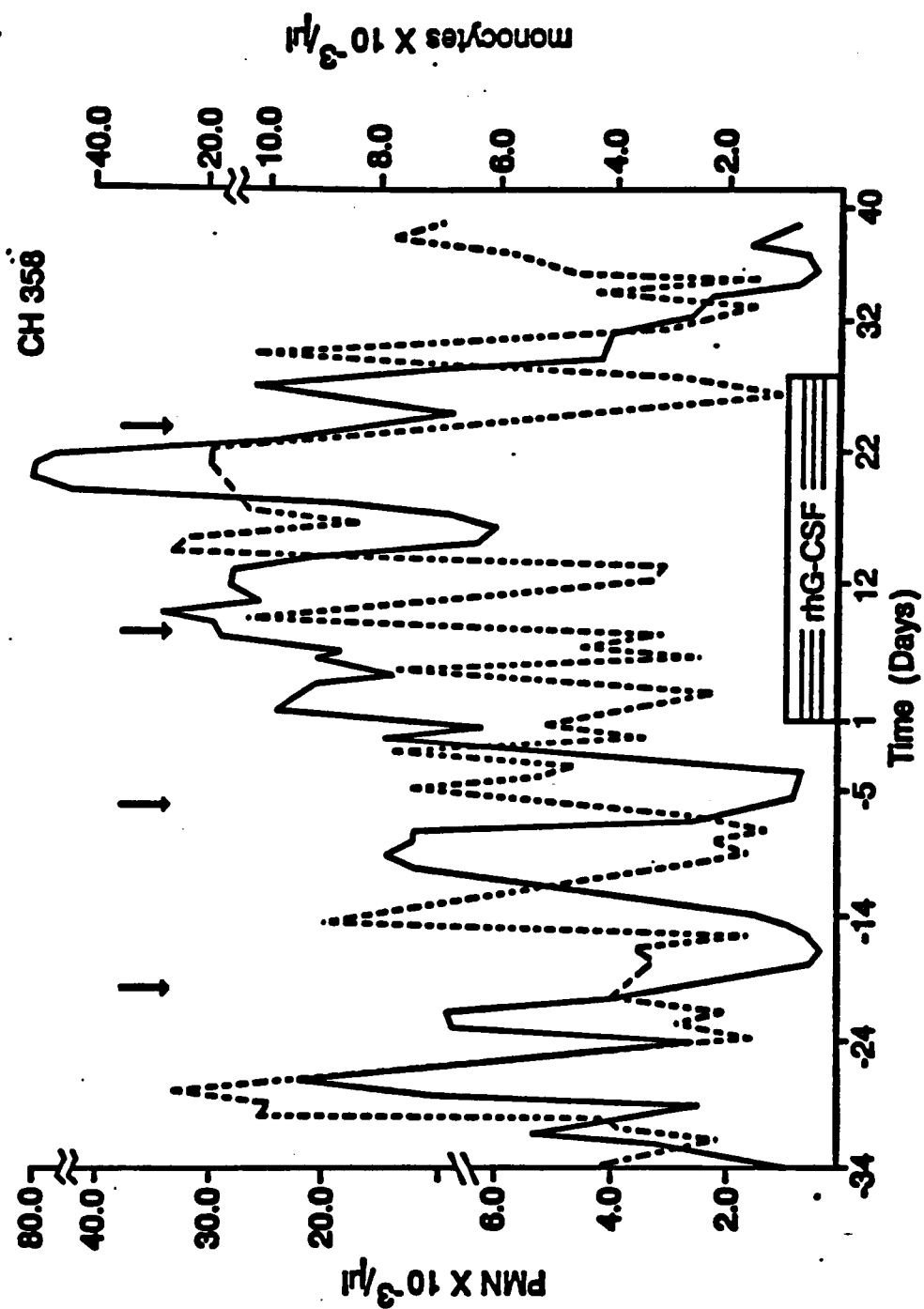


Figure 2. The effect of rhG-CSF on PMN counts (solid line) and monocyte counts (dashed line) in CH 358. Arrows indicate times of predicted neutropenia. Day 1 is the first day of rhG-CSF treatment.

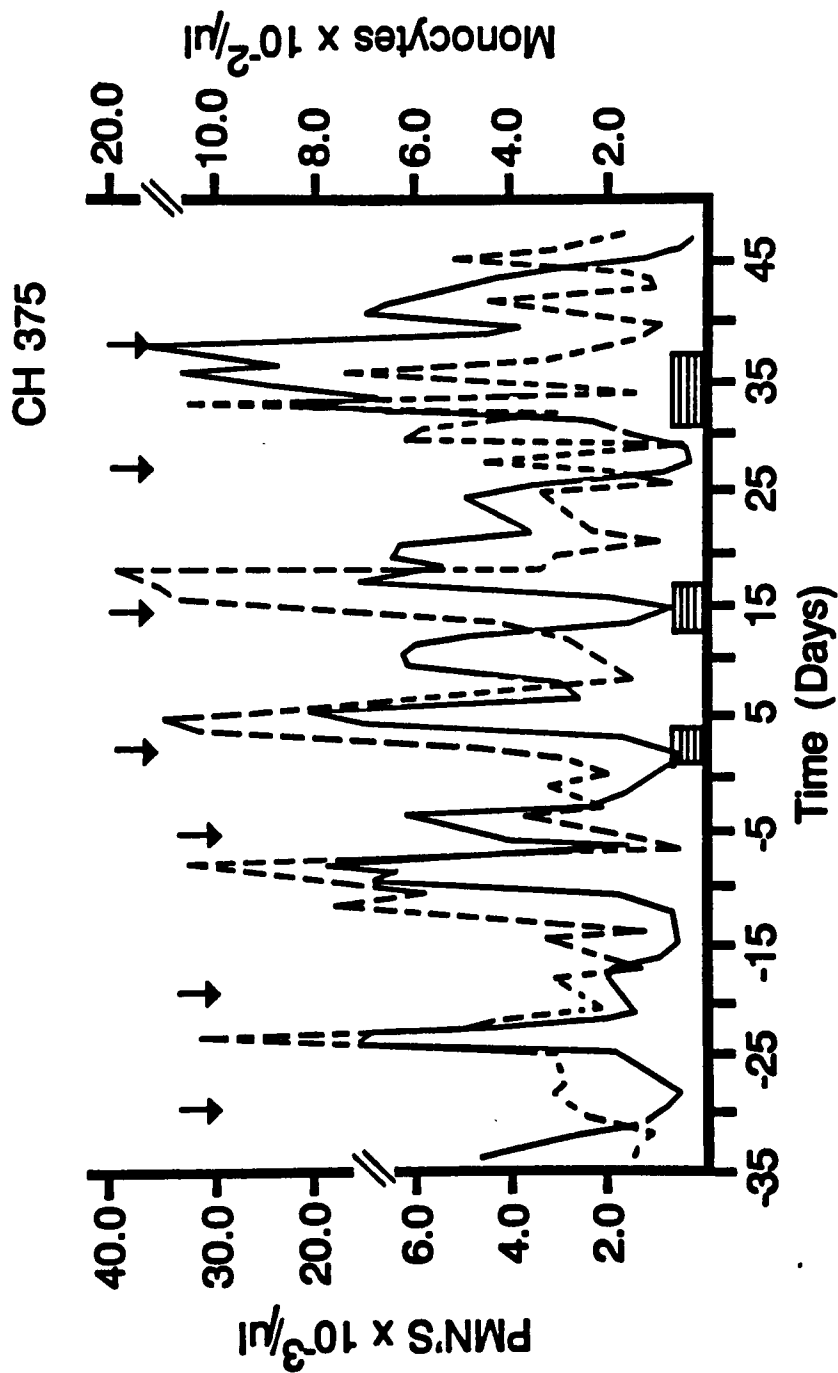


Figure 3. The effect of rhG-CSF on PMN counts (solid line) and monocyte counts (dashed line) in CH 375. Arrows indicate times of predicted neutropenia. Day 1 is the first day of rhG-CSF treatment. Note times of treatment.

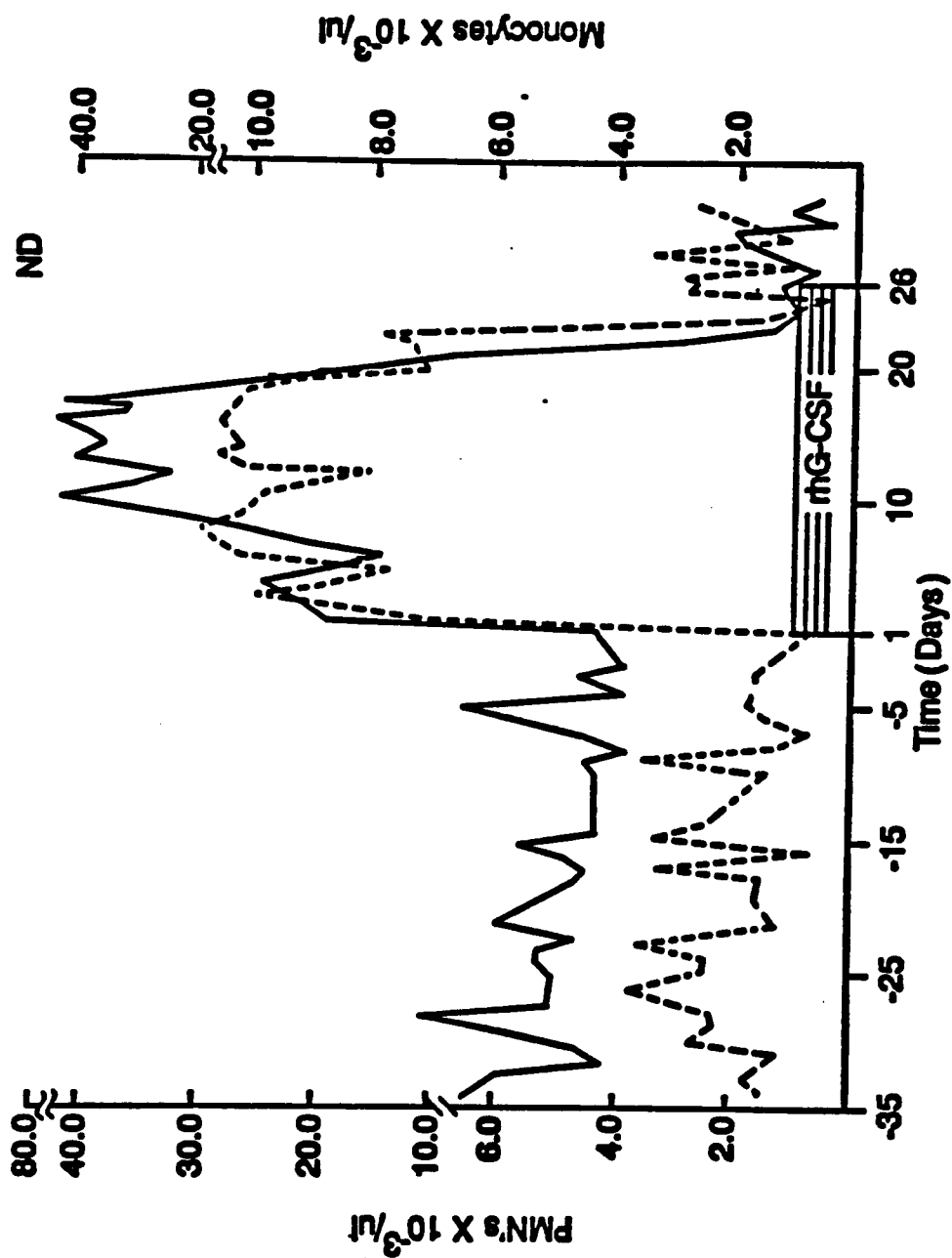


Figure 4. The effect of rhG-CSF on PMN counts (solid line) and monocyte counts (dashed line) on a normal dog. Day 1 is the first day of rhG-CSF treatment.

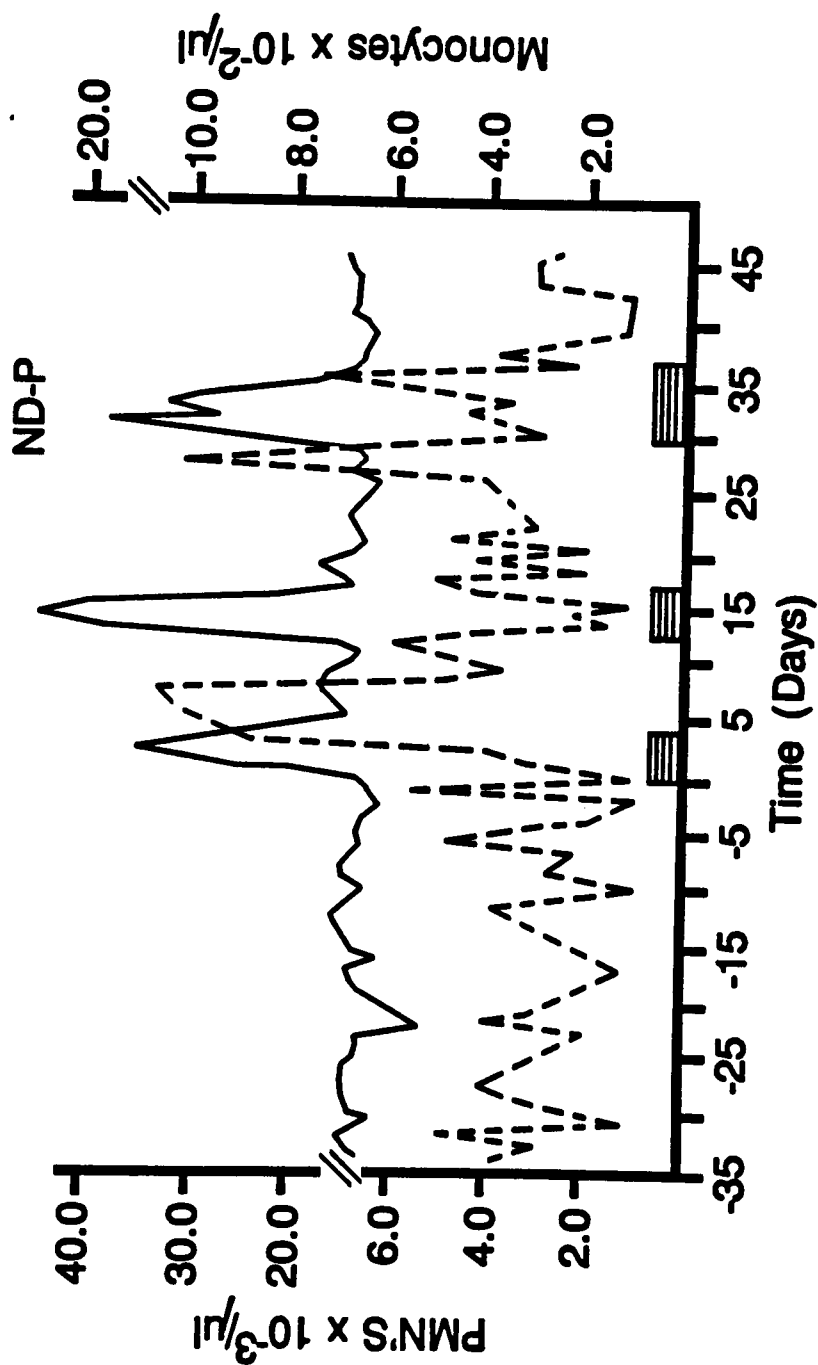


Figure 5. The effect of rhG-CSF on PMN counts (solid line) and monocyte counts (dashed line) in a pulsed treated normal dog. Day 1 is the first day of rhG-CSF treatment. Note times of treatment.

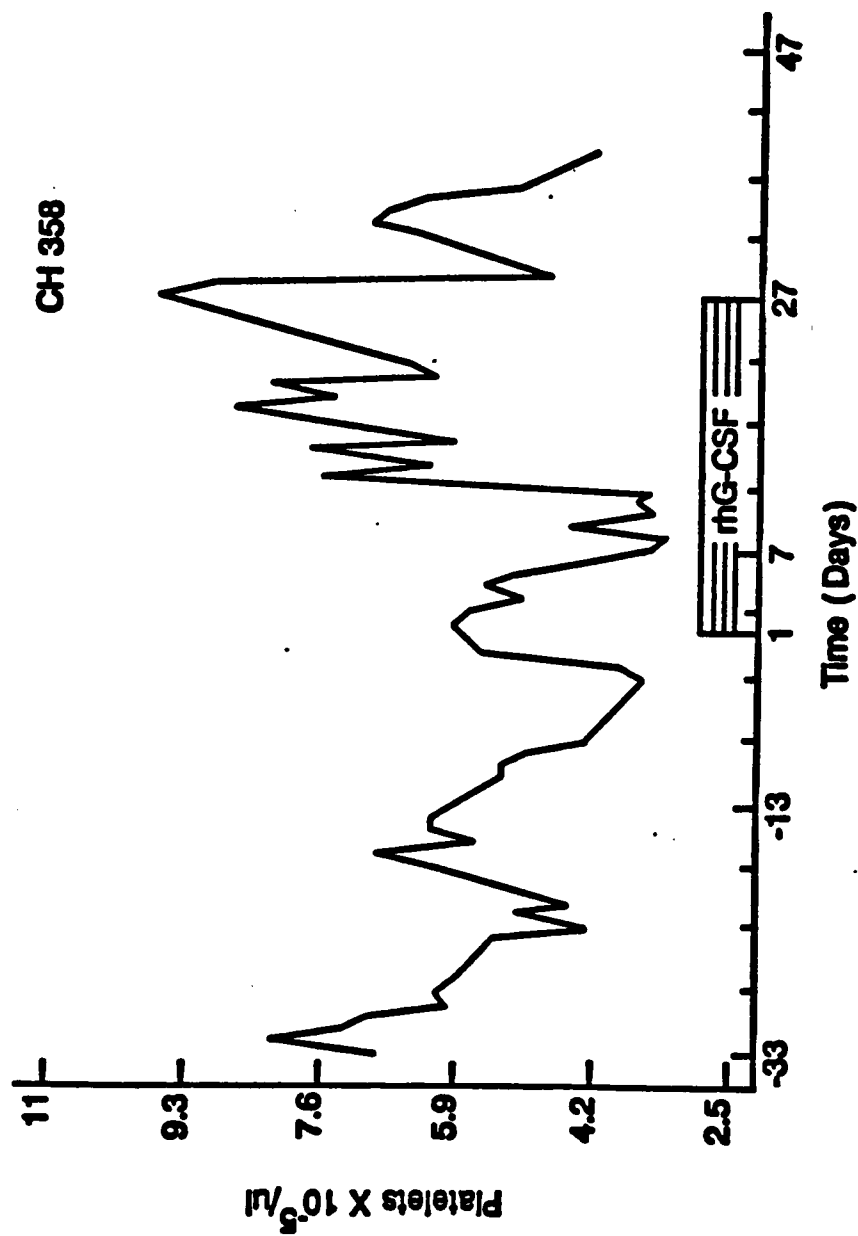


Figure 6. The effect of rhG-CSF on platelet counts of CH 358. Day 1 is the first day of rhG-CSF treatment.

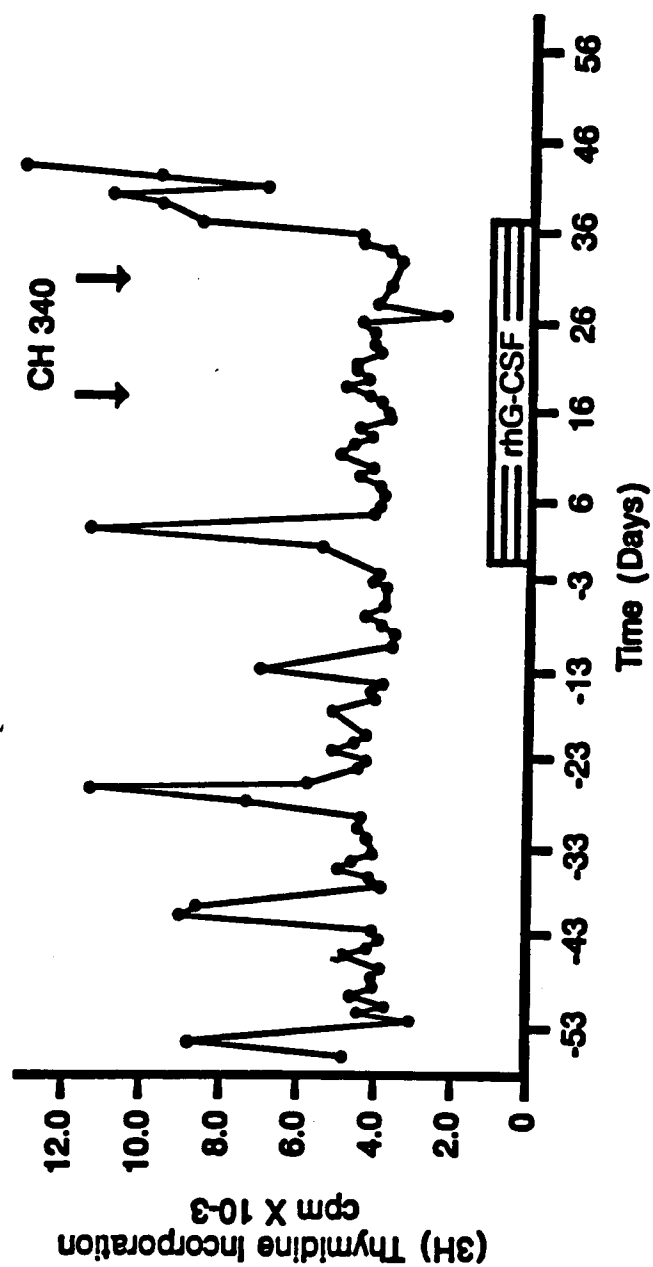


Figure 7. The effect of rhG-CSF on G-CSF activity as measured by a NFS-60 cell proliferation assay. Day 1 is the first day of rhG-CSF treatment. Arrows indicate predicted peaks of G-CSF.

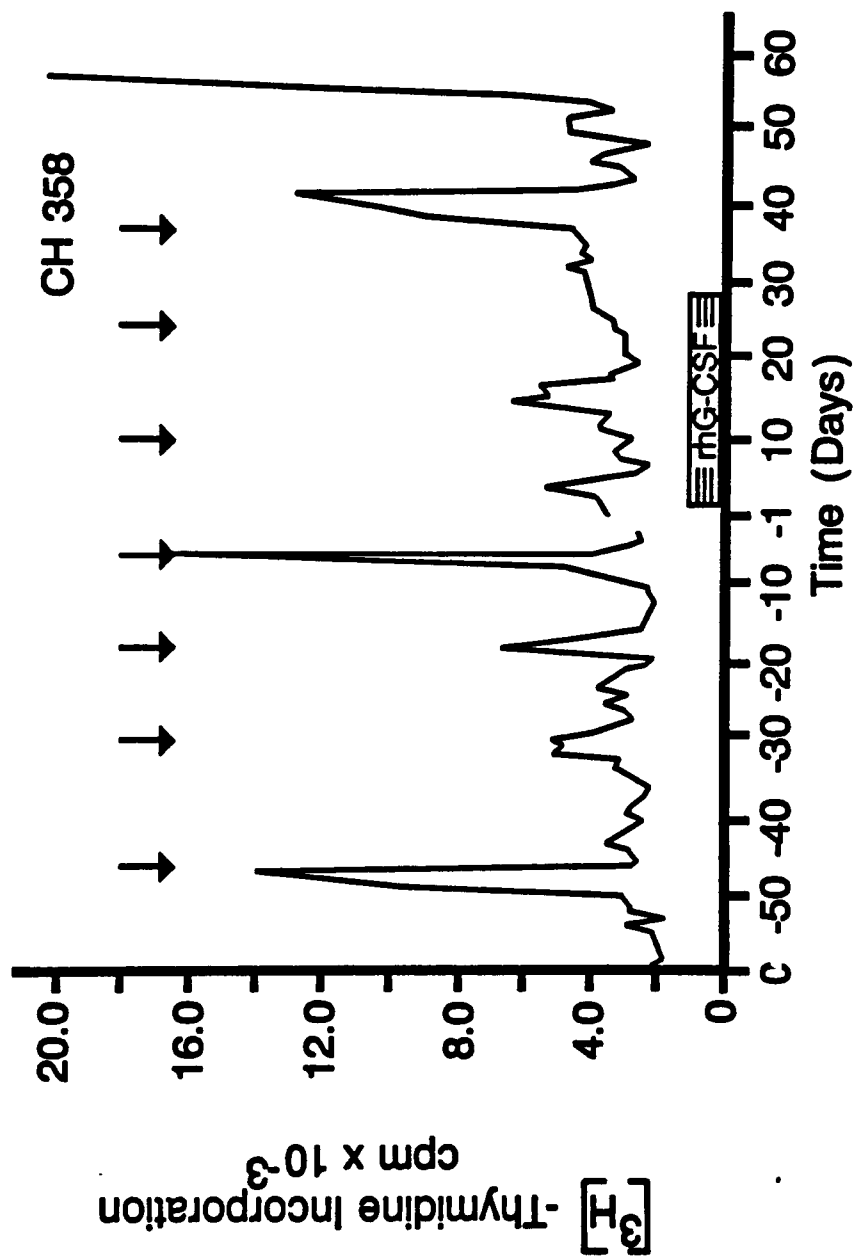


Figure 8. The effect of rhG-CSF on G-CSF activity as measured by a NFS-60 cell proliferation assay in CH dog 358. Day 1 is the first day of rhG-CSF treatment. Arrows indicate predicted peaks of G-CSF.

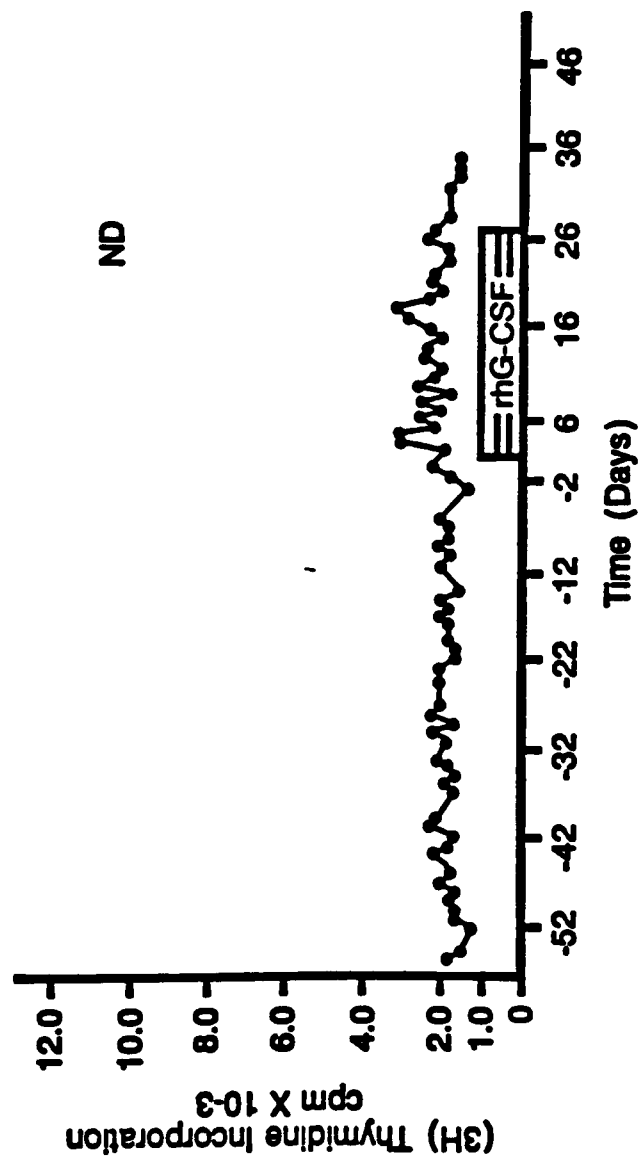


Figure 9. The effect of rhG-CSF on G-CSF activity as measured by a NFS-60 cell proliferation assay in the normal dog. Day 1 is the first day of rhG-CSF treatment.

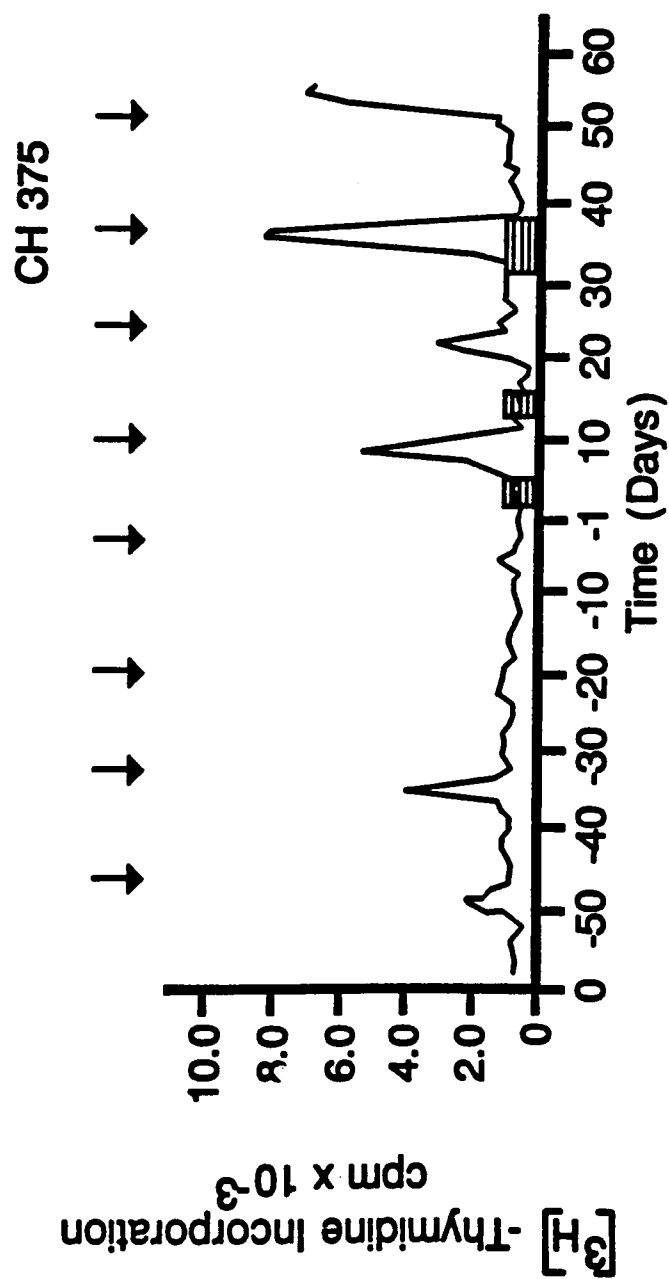


Figure 10. The effect of rhG-CSF on G-CSF activity as measured by a NFS-60 cell proliferation assay in CH 375. Day 1 is the first day of rhG-CSF treatment. Note the treatment times.

VITA

Helen Lynn Nolan Pratt was born in Memphis, Tennessee on August 20, 1962. She attended elementary schools in that city and graduated from Bishop Byrne High School in June 1980. The following fall she entered the University of Tennessee at Martin. The winter of 1983 she transferred to the University of Tennessee at Knoxville where she received a Bachelor of Arts degree in chemistry in May 1984. The following month she began work as a laboratory technician in the Endocrinology Lab at the University of Tennessee College of Veterinary Medicine. In the winter of 1987 she entered the graduate program in comparative and experimental medicine at the University of Tennessee at Knoxville. She received a Master of Science degree in May 1989.