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(57) **Abrégé/Abstract:**

Canine granulocyte macrophage colony stimulating factor (caGM-CSF) corresponding to that found in canine serum and/or tissues, structural variants thereof, genes that encode these materials, related expression vectors and cells, recombinant methods for making caGM-CSF, and veterinary treatments therewith.



CANINE GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTORAbstract of the Disclosure

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CANINE GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR

This invention was made with government support under grants CA 15704 and DK 42716 awarded by the National Institutes of Health. The government has certain rights in the invention.

5

The present invention relates to canine granulocyte macrophage colony stimulating factor (canine or caGM-CSF herein) corresponding to that found in canine serum and/or tissues. In other aspects, the invention covers, *inter alia*, structural variants of caGM-CSF, polynucleotides that encode caGM-CSF and its structural variants, expression vectors containing the polynucleotides, and cells transformed by the expression vectors.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a glycoprotein that regulates proliferation and differentiation of hematopoietic progenitors [1,2]. GM-CSF is produced by lymphocytes, monocytes, endothelial cells, and fibroblasts, as well as other cell lines [3-9]. It has broad stimulating activity *in vitro* on hematopoietic cells, alone or in combination with other hematopoietic growth factors [10-17]. It also alters the function of relatively differentiated cells, like neutrophils and macrophages, by enhancing chemotaxis and increasing oxidative metabolism, phagocytic activity, and microbicidal activity [18-24]. *In vivo* studies became possible with the isolation of the cDNA for human GM-CSF and the production of the recombinant human protein [25-32]. Animal studies and clinical trials have been completed and suggest potential therapeutic uses for recombinant human GM-CSF. However, biological and preclinical questions persist about the role of this factor in the control of hemopoiesis and the optimal use either alone or in combination with other materials to reverse perturbations of hemopoiesis secondary to a disease process or to toxicity of radiation and chemotherapy. The cloning of the cDNA for

GM-CSF from three species: mouse, cow, and man, has been reported [1, 37, and 2, respectively]. The biological activity of GM-CSF has been demonstrated to be highly species-restricted despite a significant nucleotide and amino acid sequence homology [13,37].

5 Until the work by the present inventors, no work had been reported on isolating a factor having GM-CSF activity from dogs. Several potential difficulties had to be surmounted before the present inventors succeeded in achieving the present invention. First, although GM-CSF proteins had been isolated from three other species, as mentioned above, it was unclear to what
10 extent there would be homology between these known GM-CSF materials and that from dogs. Accordingly, it was unclear whether probes based on GM-CSF proteins associated with other species would be useful in isolating an analogous material from dogs. Second, it was unclear whether canine cells could be adequately stimulated to produce enhanced amounts of messenger RNA (mRNA) for a canine
15 GM-CSF. Although it was previously reported that canine cells could be generally stimulated to produce a conditioned medium with stimulating activity on hematopoietic progenitors, it was not clear whether the resulting activity was due to canine GM-CSF protein in the medium, nor was there any indication at what level mRNA was being transcribed.

20 In spite of the above uncertainties, and other difficulties, the present inventors succeeded in isolating a full cDNA sequence corresponding to a protein having GM-CSF activity in dogs. This discovery established the basis of the present invention.

25 The present invention has several aspects. In a first aspect, the present invention relates to proteinaceous molecules having at least about 90% homology to an amino acid sequence corresponding to natural caGM-CSF and which have an activity of caGM-CSF. More specifically, this aspect of the present invention relates to isolated proteinaceous molecules comprising amino acid sequence I
30 (SEQ ID NO:1), amino acid sequence II (SEQ ID NO:2), amino acid sequence III (SEQ ID NO:3), proteinaceous molecules comprising both amino acid sequence IV (SEQ ID NO:4) and amino acid sequence V (SEQ ID NO:5), and proteinaceous molecules having at least about 90% homology to these amino acid sequences.

35 In another aspect of the present invention, it relates to isolated polynucleotide molecules encoding the proteinaceous molecules described above.

Other aspects of the present invention relate to recombinant expression vectors capable of transferring the recited polynucleotide molecules to suitable host cells, and to cells transformed by these expression vectors.

In another aspect, the present invention relates to methods of producing the
5 recited proteinaceous molecules by recombinant means.

In a further aspect of the present invention, it relates to pharmaceutical compositions comprising the recited proteinaceous molecules.

In yet another aspect, the present invention relates to specific binding partners to the recited proteinaceous molecules.

10 The above aspects of the present invention enable production of large quantities of proteinaceous molecules having canine granulocyte macrophage colony stimulating factor activity. It is contemplated that such molecules can be used, e.g., for veterinary purposes to treat dogs, as described in greater detail hereinbelow.

Embodiments of the present invention will now be described with reference to the accompanying drawings, in which:

FIGURE 1A shows Amino Acid Sequence I (SEQ ID NO:1) corresponding to natural caGM-CSF (which includes an N-terminal 17-amino acid signal peptide);

FIGURE 1B shows Amino Acid Sequence II (SEQ ID NO:2) corresponding to mature caGM-CSF without the signal peptide;

20 FIGURE 1C shows Amino Acid Sequence III (SEQ ID NO:3) corresponding to mature caGM-CSF with an N-terminal methionine residue.

FIGURE 2A shows Nucleotide Sequence I (SEQ ID NO:6), which encodes Amino Acid Sequence I (SEQ ID NO:1);

FIGURE 2B shows Nucleotide Sequence No. 2 (SEQ ID NO:7), which encodes
25 Amino Acid Sequence II (SEQ ID NO:2);

FIGURE 3 shows a polymerase chain reaction used by the inventors to construct full-length cDNA corresponding to natural caGM-CSF.

FIGURE 4 is the nucleotide sequence of full-length caGM-CSF cDNA. The start codon 'ATG' and stop codon 'TGA' (asterisks) flank an open reading frame of
30 432 base pairs with nucleotide sequence homology of 80% to the polynucleotide sequence corresponding to human GM-CSF. The nucleotide homology of the complete cDNA sequence is 65% [The amino acid homology between canine and human GM-CSF is 70%]. The highlighted 5' region which includes the start codon
35 different species (human, dog, mouse, cow). In the 3' untranslated region there is an AT rich sequence with an ATTTA motif that destabilizes the mRNA transcript.

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FIGURE 5 shows amino acid sequences of GM-CSF from four different species aligned to allow comparison and homology determination. There is an N-terminal sequence of 17 amino acids characteristic of signal sequences (arrows). Human GM-CSF is known to have two N-glycosylation sites, but canine GM-CSF has only one predicted site (asterisks). The positions of the cysteine residues are identical between canine and human GM-CSF (squares). There are regions of functional importance on human GM-CSF: amino acids 38-48 and amino acids 95-111 [47] (underlined), of which the latter has the most similarity to canine GM-CSF. Gaps (represented by dots) were introduced in sequences to maximize alignment.

In FIGURE 6, the blot was probed with ^{32}P -labeled caGM-CSF cDNA. Restriction digests of DNA (10 μg) were with Bam HI, Bgl I, Eco RI, Hind III, and PstI. Single bands were seen in BAM HI + Eco RI lanes suggesting that caGM-CSF is a single copy gene.

In FIGURE 7, marrow mononuclear cells were collected from a Ficoll-HypaqueTM gradient, and depleted of monocytes and T-lymphocytes, and cultured at 7.5×10^4 cells/plate for 10 days. Different dilutions of supernatant enriched with caGM-CSF were added to the CFU-GM assays. Negative controls were assays with heat-inactivated Fetal Calf Serum only as well as assays that had supernatant from normal COS cells (not transfected). Positive controls were assays done with both 10% post endotoxin dog serum and also with human AB plasma.

To produce FIGURE 8, four dogs received five courses of caGM-CSF subcutaneously twice daily for fourteen days. Doses ranged from 1 μg -50mg/kg/day. The dog that received 1 μg /kg/day (•—•) received a second fourteen day course of treatment of 10 μg /kg/day (○—○) after a fourteen day period of rest. Peripheral blood counts for neutrophils, lymphocytes, monocytes and eosinophils were obtained before, during and after treatment. Increases were noted in all categories, depending on the dose of caGM-CSF received.

FIGURE 9 shows platelet counts for 4 dogs that received 5 courses of caGM-CSF subcutaneously twice daily for 14 days. The dog that received 1 μg /kg/day (•—•) received a second 14 day course of treatment of 10 μg /kg/day (○—○) after a 14 day period of rest. Decreased platelets were noted within 24 hours of initiating treatment.

The present inventors have isolated and cloned the cDNA for canine GM-CSF. Using this cDNA, a polypeptide corresponding to canine GM-CSF has been expressed by transformed cells, and may now be used therapeutically and for
5 *in vitro* and *in vivo* studies. The isolated cDNA is an 850 base pair sequence (SEQ ID NO:6) which encodes the naturally occurring protein having 144 amino acids, which includes the signal peptide of 17 amino acids.

The subsequent discussion herein will be facilitated by the following pertinent definitions:

10 "Proteinaceous molecule" - Generally, any molecule made up of a plurality of amino acids. The term is broad enough to include peptides, oligopeptides, and proteins. Typically, the amino acids in the proteinaceous material will be selected from the 20 naturally occurring amino acids. However, amino acid analogs and derivatives could also be included in the proteinaceous molecule. The
15 proteinaceous molecule will usually be made up of about the same number of amino acids contained in naturally occurring canine GM-CSF (i.e., about 144 amino acids). In addition, however, the proteinaceous molecule may have a greater or lesser number of amino acids (for example, 144 ± 30 amino acids), as long as the molecules retain an activity of caGM-CSF. This activity need not be
20 quantitatively the same as the activity of the natural protein, and may be more or less than that activity, as long as it is measurable by an assay of caGM-CSF activity (see below). Preferably, such molecules will possess at least about 50% of the activity of the naturally occurring caGM-CSF without the signal peptide (Amino Acid Sequence II; SEQ ID NO:2). In some hosts, expression of recombinant
25 caGM-CSF will result in a protein having an N-terminal methionine residue. Amino acid sequences containing such a residue on the N-terminus thereof are also within the scope of the present invention.

By "activity of caGM-CSF" is meant any activity that is measurable by an *in vivo* or *in vitro* GM-CSF assay. Qualitatively, the activity will generally be one
30 that is possessed by the naturally occurring canine GM-CSF protein. A preferred *in vivo* assay for measuring granulocyte macrophage colony stimulating factor activity is referred to as a granulocyte macrophage colony forming unit (CFU-GM) assay. This assay involves measuring the number of colonies of granulocytes and macrophages from dogs formed *in vitro* in response to contact with a test
35 substance. A typical assay procedure is described in Schuening et al. [38]. Other known assays may also be used. For example, *in vivo* assays of caGM-CSF activity can involve measuring marrow cellularity, and/or peripheral counts, in response to administration of a test substance to a dog.

By "isolated" is meant that a substance is substantially free from normally occurring impurities or other molecules, especially other proteinaceous molecules, salts, other cellular constituents, etc. Isolation may typically be carried out by standard methods in the art of nucleotide, peptide and/or protein purification or synthesis.

"Polynucleotide sequences" encompass both DNA- and RNA-containing molecules. The DNA molecules will preferably be intronless sequences (i.e., cDNA), but can contain enhancer sequences, termination sequences, and the like, to facilitate or increase expression in a particular host. Messenger RNA (mRNA) molecules are also encompassed by the term.

By "recombinant expression vector" is meant a vector (e.g., a plasmid or λ phage) that is capable of transferring polynucleotide sequences contained therein into cells of a host organism for expression of the transferred sequences. The sequences are operably linked to other sequences capable of effecting or modulating their expression. Such expression vectors must be replicable in a host organism. For example, any DNA sequence that is capable of effecting expression of a specified DNA sequence disposed therein is included in this term as it is applied to the specified sequence.

The "cells" that may be transformed by way of the vectors described above are those that are capable of expressing the polynucleotide sequences that have been transferred by the vector. Culturing conditions for such cells may be those standard in the art of recombinant protein production.

"Specific binding partners" are molecules that are capable, on a molecular level, of recognizing and interacting with the proteinaceous or polynucleotide molecules described herein; included within this term are immunological binding partners such as antibody molecules, antigen-binding fragments of antibodies (e.g., Fab and F(ab')₂ fragments), single chain antigen-binding molecules, and the like, whether produced by hybridoma or rDNA technology. Other proteinaceous or non-proteinaceous binding partners are also included within the broad term.

30 Proteinaceous Molecules

The proteinaceous molecules of this invention will now be described in greater detail. Amino Acid Sequence I (SEQ ID NO:1) corresponds to the naturally occurring canine peptide having 144 amino acids. The first 17 amino acids, extending from Met-1 to Ser-17 are believed to be a signal peptide, which is presumably cleaved by a signal peptidase *in vivo* or during recombinant expression using many host cells. Amino Acid Sequence II (SEQ ID NO:2) has a sequence

corresponding to Amino Acid Sequence I without the signal peptide sequence. Thus, it corresponds to mature caGM-CSF.

5 Amino Acid Sequences III (SEQ ID NO:4) and IV (SEQ ID NO:5) correspond to amino acids 38-48 and 95-111, respectively, in Amino Acid Sequence I. These amino acid sequences are believed to be important functional regions of caGM-CSF. Amino acid sequences containing both of these sequences, preferably separated by approximately 46 amino acids (as in the natural sequences) are also part of the present invention.

10 The family of caGM-CSF factors provided herein also includes proteinaceous molecules in which one or more of the amino acids in the above-recited amino acid sequences has been deleted, modified, or changed to another amino acid. Site directed mutagenesis is a preferred technique enabling conversion of one amino acid to another. For example, one or more of the cysteine residues may be changed to another amino acid such as serine. One possible reason for such a
15 change would be to eliminate one or more unwanted disulfide bonds. See, for example, U.S. Patent No. 4,518,584.

Other contemplated specific changes in the natural amino acid sequences involve modification of the asparagine glycosylation site. Modification of the asparagine or one or both of the subsequent two amino acids (asparagine and
20 serine in Amino Acid Sequences I and II) can eliminate glycosylation at the modified site. Thus, for example, the asparagine could be changed to a glutamine, thereby eliminating glycosylation at the site. See, for example, A. Miyajima et al., EMBO J., 5(6):1993 (1986).

The modifications of the amino acid sequences described above that form a
25 part of the present invention are those that result in proteinaceous molecules having an activity of canine GM-CSF. Such activity may be determined using the assays described above, or equivalent standard assays. The family of proteinaceous molecules of the present invention will also generally have at least about 90% homology to amino acid sequences I, II, or III which means that several
30 of the amino acids in each sequence may be deleted, modified, or changed (assuming the resulting proteinaceous material retains at least some activity in common with canine GM-CSF).

The proteinaceous molecules of the present invention may further be labeled by attachment to a detectable marker substance (e.g., radiolabeled with ¹²⁵I) to
35 provide reagents useful *in vitro* or *in vivo*.

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Polynucleotide Sequences

The present invention is also directed to polynucleotide sequences. Preferred polynucleotide sequences are DNA molecules that encode the proteinaceous molecules of the present invention, described above. A preferred
5 DNA sequence (SEQ ID NO:6) is the one shown in FIGURE 2A herein. The novel cDNA sequence illustrated in FIGURE 2A included in a plasmid has been deposited on November 20, 1990 in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD and given accession No. ATCC 40921. The cDNA of FIGURE 2A is described in greater detail in the Examples section below.

10 It is to be recognized that more than one DNA sequence can encode the same amino acid sequence, due to the degeneracy of the genetic code; all such sequences are encompassed by the polynucleotide sequences described herein.

The method of obtaining the cDNA of FIGURE 2A is described in the Examples Section below.

15 Polynucleotide products of the present invention may be labeled with detectable markers (such as radiolabels and non-isotopic labels such as biotin) and employed, for example, in DNA hybridization processes to locate the canine gene position and/or the position of any related gene family in a chromosomal map. They may also be used for identifying canine gene disorders at the DNA level and
20 used as gene markers for identifying neighboring genes and their disorders.

Expression Vectors, Hosts, and Recombinant Methods

In general terms, the production of a recombinant canine GM-CSF typically involves the following:

Initially, a DNA encoding the mature protein, the preprotein (containing the
25 signal peptide), or a fusion of the caGM-CSF protein to an additional sequence which does not destroy its activity or to an additional sequence cleavable under controlled conditions (such as treatment with a peptidase) to give an active protein, is obtained. If the sequence is uninterrupted by introns, it is suitable for expression in any host. If there are introns, expression is possible in mammalian
30 or other eukaryotic systems capable of processing them.

Next, the isolated coding sequence is placed in operable linkage with suitable control sequences in a replicable expression vector. The vector is then used to transform a suitable host and the transformed host cultured under favorable conditions to effect the production of recombinant canine GM-CSF. Optionally,
35 the caGM-CSF is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in instances where some impurities can be tolerated. For example, for *in vitro* cultivation of cells from

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which a lymphokine factor will be isolated for administration to a subject, complete purity is not required. However, direct use in therapy by administration to a subject would, of course, require purification of the canine GM-CSF produced.

5 The constructions for expression vectors operable in a variety of hosts are made using appropriate control sequences, etc., as set forth below. Generally, prokaryotic, yeast, or mammalian cells are useful as hosts. Eukaryotic cells, particularly mammalian cells, are preferred when a processing step (e.g., intron excision, glycosylation, etc.) is desired.

10 A variety of prokaryotic cells known to those of ordinary skill in this art may be utilized. A few exemplary strains include: *E. coli.*, *Bacillus subtilis*, and various strains of *Pseudomonas*.

15 In addition to bacteria, eukaryotic microbes, such as yeast, may also be used as host. Laboratory strains of *Saccharomyces cerevisiae*, bakers yeast, are most commonly used.

20 It is also possible to express genes encoding polypeptides in eukaryotic host cell cultures derived from multi-cellular organisms. Useful host cell lines include Vero, HeLa, COS, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and later promoters from Simian Virus 40 (SV40) or other viral promoters such as those derived from polyoma, adenovirus 2, bovine papilloma virus, avian sarcoma viruses, immunoglobulin promoters and heat shock promoters. Enhancer regions may also be included as desired.

25 Other examples of hosts, vectors, enhancers, promoters, etc., may be found in the following exemplary U.S. Patents: 4,810,643; 4,766,075; and 4,847,201.

Pharmaceutical Compositions

30 Also contemplated by the inventors as part of this invention are pharmaceutical compositions comprising effective amounts of proteinaceous molecules as described herein. The proteinaceous molecules will typically be combined with suitable diluents, agents and/or carriers useful in GM-CSF therapy. The proteinaceous molecules of the present invention may be incorporated as the sole active ingredient in such pharmaceutical compositions, or
35 they may be combined with other active agents, such as other hematopoietic factors or drugs useful in treatment of hematopoietic disorders, such as aplastic anemia. Other lymphokines (e.g., IL-3, M-CSF, IL-1, IL-6, G-CSF, Steel locus

factor) may also be incorporated in these compositions. The combination of such active ingredients (proteinaceous molecules of this invention and other active materials) may result in synergistic activity for a particular purpose, so that reduced quantities of active ingredient may be made possible.

5 Uses of the Proteinaceous Materials and Pharmaceutical Compositions

Granulocyte-macrophage colony stimulating factor has been demonstrated to have significant effects on both the hematopoietic and immunologic systems in humans and mice. Applications for canine GM-CSF as therapy in dogs are several. It could be used as an adjunct to existing chemotherapy or more intensive
10 chemotherapy regimens for various oncologic disorders, especially lymphoma, to lessen the hematopoietic toxicity. It also could be used as an agent to prime host defenses against parasitic, bacterial or viral infections, with or without the additional of antibiotics. Antibiotics are a selective force in generating antibiotic-resistant microbes. Use of canine GM-CSF especially as prophylaxis,
15 for instance, after surgery, may be effective in reducing the infection rates as well as the use of antibiotics. Certain hematopoietic conditions in the dog that are disease-related and also associated with neutropenia may also be amenable to therapy with canine GM-CSF.

The advantages of the canine species factor as therapeutic agent rather than
20 the human-derived factor are several. The most cogent is the increased potency. GM-CSF is also an agent with multiple roles and one cannot be sure of the full-spectrum of activity with a factor derived from another species. The species-specific factor is less likely to generate an immune response with the formation of neutralizing antibodies which could limit therapeutic administration.

25 A representative dosage is 30-50 μ g/kg/day diluted in 0.9% NaCl and administered subcutaneously. Dosages and indications for treatment would be refined as studies progressed.

Specific Binding Partners

Specific binding partners directed to the proteinaceous molecules and
30 polypeptides of the present invention may be generated by any standard technique known to those of ordinary skill. Preferred specific binding partners are immunological binding partners such as intact antibodies and fragments thereof. The immunological binding partners are preferably monoclonal antibodies directed to a specific antigen, which are prepared by standard techniques of monoclonal
35 antibody production.

These specific binding partners may be utilized, for example, to purify the proteinaceous materials or polynucleotides of the present invention. Specific

binding partners in labeled form may be utilized to indicate the presence of the proteinaceous molecules or polynucleotides of the present invention. In one preferred embodiment, a specific binding partner of a polynucleotide of the present invention may be utilized in labeled form to locate the natural gene on a
5 chromosome.

The invention now being generally described, the same will be better understood by reference to certain specific examples which are included herein for illustrative purposes only and are not intended to be limiting of the present invention, except where so stated.

10

Examples

Isolation of the Full-Length caGM-CSF cDNA

To obtain a T cell lymphocyte enriched population, peripheral blood and splenic mononuclear cells were isolated from a Ficoll-Hypaque gradient. They were then cultured for 48 hours in RPMI-1640 medium (M.A. Bioproducts) supplemented with 5% fetal calf serum (Gibco) in the presence of 1%
15 phytohemagglutinin (Difco) and 5% pokeweed nitrogen (Gibco). Blast morphology was determined microscopically in all cultures to confirm stimulation of T-lymphocytes prior to RNA extraction on a cesium chloride gradient [39]. A cDNA library in a Lambda Zap cloning vector (Stratagene) [40] was constructed
20 and packaged. The library was screened with the cDNA for the coding region of hGM-CSF (Genetics Institute). Probes were radiolabeled utilizing random hexamers and [³²P] α deoxy-cytidine triphosphate [41]. Hybridization was performed in a buffer containing 35% (V/V) formamide at 37°C over night. Nylon filters (Amersham) were washed at room temperature in 2xSSPE, 0.5% SDS and
25 then 0.5xSSPE for 20 minutes each. Plasmids (pBluescript SK(-)) containing the canine GM-CSF cDNA were subcloned from the Lambda Zap phage by an *in vivo* excision process in the presence of helper virus [40].

A library of canine genomic DNA, partially digested with MboI and ligated into EMBL4 was obtained (Clontech). It was screened with a radiolabeled cDNA
30 [41] for canine GM-CSF. Hybridization of the filters was done with 50% (V/V) formamide at 42°C and the washes were performed at 42°C. The genomic DNA inserts were excised by Sal I and then restriction mapped. A restriction fragment that hybridized with the caGM-CSF cDNA was subcloned into pBluescript SK(-).

A full-length cDNA for caGM-CSF was made by first producing a template
35 for Exon I from the gene through amplification by the polymerase chain reaction (PCR) [42]. Then, a second PCR was done with 2 DNA templates - the Exon I amplicon and the partial cDNA for caGM-CSF. These two templates had

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overlapping complementary sequences 80bp long. The primer-linkers (20bp/Bam HI) were oligonucleotides with complementation to a region at the start codon of Exon I and a region at the stop codon of the partial cDNA. This created the full-length 432bp coding region for the cDNA of caGM-CSF flanked by sequences for Bam HI restriction sites. See FIGURE 4.

- 5 Clones were sequenced in pBluescript SK(-)TM using the dideoxynucleotide method [43] as described in the SequenaseTM (United States Biochemical) protocol for double-stranded DNA. All pBluescript SK(-)TM clones with the cDNA or genomic fragment inserts were initially sequenced from the flanking T3 and T7 primers. Sequence analysis was done with GeneproTM Version 4.2 software (Riverside Scientific Enterprises).

Genomic DNA Analysis

- Genomic DNA was isolated from canine peripheral blood mononuclear cells as has been described [39]. Restriction digests were performed and DNA was electrophoresed in a 1.0% agarose gel. Southern transfer was done onto a nylon
15 filter and hybridized with a ³²P-labeled canine GM-CSF probe.

Protein Expression for In Vitro Analysis

- The full-length caGM-CSF cDNA with flanking Bam HI sites was subcloned into a mammalian expression vector, pCMV. Correct orientation of the insert was determined by Pst I restriction digest for which there was one unique site in the
20 cDNA. The pCMV/CAGM vector was sequenced with sequence-specific primers for caGM-CSF. The vector was then transfected into COS cells by the calcium phosphate precipitation method [44]. Cells were grown as described [45], and supernatant harvested at 72 hours and 120 hours.

- Canine CFU-GM assays were performed as described [38] with dilutions of
25 supernatant from COS cells transfected with pCMV/CAGM. Supernatant harvested from COS cells that had not been transfected served as one of the negative controls. Positive controls were CFU-GM assays done in the presence of post-endotoxin dog serum or human AB plasma which had been screened for activity in canine CFU-GM colony assays. Marrow mononuclear cells collected
30 from a Ficoll-HypaqueTM gradient were depleted of monocytes and T-lymphocytes and cultured at 7.5×10^4 cells/plate for 10 days at 37°C in a humidified 10% CO₂ incubator in 35 mm plastic petri dishes containing 2 ml of agar medium. Agar
medium consisted of an equal volume mixture of 0.6% (wt/vol) Bacto-AgarTM (Difco) and double-strength Dulbecco's modified Eagle's medium (Gibco) containing 40%
35 (V/V) heat-inactivated FCS.

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In Vivo Analysis

Four dogs were treated with caGM-CSF expressed in *E. coli* (protein expressed and provided by Amgen, Thousand Oaks, CA). Doses were administered subcutaneously twice daily. The first dog (C918) was given 1 μ g/kg/day caGM-CSF for 14 days, then a 14-day rest with no treatment and then another course of 10 μ g/kg/day caGM-CSF for 14 days. The other three dogs were treated with 10, 30 and 50 μ g/kg/day caGM-CSF for 14 days. Baseline peripheral blood counts were established by two measurements prior to administration and then counts were followed daily. Marrow aspirations and biopsies were obtained as previously described [38] before and after 14 days of treatment. Platelet survival studies with ⁵¹Cr-labeled autologous platelets were done on dogs which received the 10 and 50 μ g/kg/day doses [46].

All dogs were dewormed and vaccinated for rabies, distemper, leptospirosis, hepatitis and parvovirus. They were housed in an American Association for Accreditation of Laboratory Animal Care approved facility in standard indoor runs and provided commercial dog chow and chlorinated tap water *ad libitum*. Animal holding areas were maintained at 70° $\pm$ 2°F with 50% $\pm$ 10 relative humidity using at least 15 air changes/hour of 100% conditioned fresh air. The dogs were on a 12-hour light/dark full spectrum lighting cycle with no twilight. The protocol for this study was approved by the internal animal use committee of the Fred Hutchinson Cancer Research Center.

Results

Isolation of Full Length caGM-CSF cDNA Clone

The cDNA library that was constructed from canine peripheral blood and splenic mononuclear cells had a titre of 3 x 10¹⁰ plaque forming units. Only 3 unique clones were isolated, all of which were incomplete cDNAs of caGM-CSF. After all clones were characterized by sequencing, it was evident that they were all incomplete at the 5' end. To obtain the remaining sequence, the incomplete cDNA for caGM-CSF was ³²P-labeled and used to screen a canine genomic library.

A genomic clone was isolated that had a 12kb insert. A 4.5kb Bam HI-Sal I restriction fragment from the clone was identified as having the caGM-CSF gene. This fragment was subcloned into pBluescript Sk(-) for sequencing. A primer derived from the sequence of the 5' end of the longest incomplete cDNA previously isolated allowed sequencing of Exon I, completing the sequence for the full-length cDNA.

A full-length cDNA for the coding region of caGM-CSF was produced by PCR amplification of Exon I and the longest incomplete cDNA clone. The PCR product of this reaction was subcloned into pCMV, a mammalian expression vector with a CMV promoter. Four of these subclones were sequenced. Nucleotide
5 misincorporation from the amplification reaction was noted in 3 of the 4 subclones. Each of these three subclones had 1, 2 and 5 base pair misincorporations, some of which were common to at least 2 subclones. The fourth clone had no misincorporations and was used to express the peptide for *in vitro* studies.

10 The nucleotide sequence for caGM-CSF is shown in FIGURES 2A and 4. There is an open reading frame beginning with the start codon "ATG" and ending at the stop codon "TGA", which is 432 bp in length and has an 80% sequence homology with the polynucleotide sequence corresponding to hGM-CSF. There is a
15 17bp sequence which includes the start codon that is common to all four species from which GM-CSF has been isolated. The 3' region includes AT-rich sequences containing an ATTTA sequence motif. This feature is noted on other lymphokines, cytokines and proto oncogenes.

A comparison of all known amino acid sequences of GM-CSF is summarized in FIGURE 5. The degree of homology between GM-CSF of dog and that of
20 human, cow and mouse is 70%, 71%, and 53%, respectively. The comparison indicates that a 17 amino acid region typical of signal sequences precedes Ala-18 at the amino terminus of the mature polypeptide. The positions of the cysteine residues which are sites of disulfide bonds stabilizing secondary structure are conserved. There is one possible N-glycosylation site on caGM-CSF, compared to
25 two on each of the other species. The N-glycosylation sites on murine GM-CSF are positioned differently, as compared to the other three types. Of the two regions associated with function in hGM-CSF [47,48], the stretch from amino acids 95-111 appears to be the most highly conserved portion to caGM-CSF.

Analysis of Canine Genomic DNA for GM-CSF

30 Canine genomic DNA was restriction digested and a Southern blot probed with ³²P-labeled caGM-CSF (FIGURE 6). Single hybridizing bands were noted in lanes with DNA digested with Bam HI + Eco RI. This suggests that the gene for canine GM-CSF is single copy as has been shown for other species. In other lanes there were two hybridizing bands suggesting single internal restriction sites in the
35 gene for Bgl I, Hind III, and Pst I.

Protein Expression and *In Vitro* Activity of CaGM-CSF

Transfection of COS cells for transient expression was done with the mammalian cell expression vector, pCMV/CAGM. Supernatant was collected at 72 and 120 hours after transfection for analysis of activity. Biologic activity was assessed by adding supernatant from transfected COS cells enriched for caGM-CSF at different dilutions to canine CFU-GM assays (FIGURE 7). Culture plates without any addition of growth factor or with supernatant from COS cells that had not been transfected served as two negative controls. Plates with 10% post-endotoxin dog serum or with human AB plasma were the positive controls. The number of CFU-GM colonies formed in the caGM-CSF containing plates was 4- to 7-fold greater than the baseline established with the 2 negative controls and equivalent to the numbers of colonies obtained with addition of post-endotoxin dog serum.

In Vivo Activity of caGM-CSF

Four normal dogs were treated with caGM-CSF to assess the *in vivo* effect on hematopoiesis. Three dogs had a single dose schedule of 10 μ g/kg/day, 30 μ g/kg/day and 50 μ g/kg/day for 14 days, administered subcutaneously twice daily. There was an increase in the peripheral blood neutrophil count, as well as the lymphocyte and monocyte count compared to pre-treatment baseline (FIGURE 8). There was a suggestion of a rise in the absolute eosinophil count in the peripheral blood but this was more variable, being unremarkable in the dog receiving the 50 μ g/kg/day dose. Day 14 marrow after treatment appeared more hypercellular at the higher doses schedules, and there was evidence of increased mitotic activity. The fourth dog had two treatments in series of 1 μ g/kg/day caGM-CSF administered for 14 days, followed by 14 days of rest and then 10 μ g/kg/day caGM-CSF for another 14 days. At 1 μ g/kg/day, the dog had a slight elevation in neutrophil count. When the 10 μ g/kg/day dose was administered, the peripheral blood neutrophil count increased 6- to 7-fold when compared to the pre-treatment baseline. A residual effect of the first dose of caGM-CSF 14 days previously could explain the marked increase in peripheral blood counts compared to other animals that received similar or higher doses.

The platelet count decreased in all the animals within twenty-four hours of the start of treatment (FIGURE 9). Platelets continued to decrease for 4-8 days of treatment before stabilizing at levels which were dose dependent. Platelet counts returned to normal within several days of stopping the caGM-CSF, starting to increase within 24-48 hours after the last dose. Peripheral blood smears demonstrated large platelets with no morphologic abnormalities similar to the

clinical syndrome of idiopathic thrombocytopenia purpura. Examination of the marrows showed that megakaryocytes were normal to increased in frequency. Platelet survival studies with ^{51}Cr -labeled autologous platelets were done in two dogs receiving the dose of $10\mu\text{g/kg/day}$ and $50\mu\text{g/kg/day}$ of caGM-CSF. Studies
5 were done to establish the normal survival curves of platelets in each animal as well as in the first and second week of treatment. These studies showed a decreased survival of platelets from about 5 to 6 days normally to 1.5 to 2.0 days.

Discussion

The cDNA for canine GM-CSF has been cloned and the polypeptide expressed
10 for *in vitro* and *in vivo* studies. The nucleotide sequence homology of the open reading frames between caGM-CSF and hGM-CSF is 80%. There is a 5' stretch 17bp in length including the start codon which is the same in all four of the GM-CSF cDNAs from different species currently reported [1,2,37]. This may facilitate the cloning of GM-CSF cDNAs from other species. In the 3'
15 untranslated region, there is an AT-rich region with an ATTTA motif which has been described as destabilizing mRNA transcripts that may be important for regulation of GM-CSF. This motif is highly conserved in GM-CSF cDNA from different species.

The gene for canine GM-CSF was shown to be a single copy in a Southern
20 blot of restriction digested canine genomic DNA. The high degree of nucleotide and amino acid sequence homology between canine and human GM-CSF as well as the observation that this is a unique sequence in canine genomic DNA suggest that this molecule is in fact the canine homologue to human GM-CSF.

The *in vitro* biologic activity of the protein was confirmed by CFU-GM
25 assays of canine marrow. The activity of caGM-CSF expressed from COS cells was similar to that of 10% post endotoxin dog serum in canine CFU-GM assays. Canine GM-CSF has also been demonstrated to stimulate human GM-CSF dependent cell lines such as Tall 101, but other human GM-CSF dependent cell lines may not be as responsive (Dale, D.). Human GM-CSF also stimulates *in vivo*
30 canine hematopoiesis [38]. The cross-reactivity may be explained by the high degree of homology, especially in the region 95-111 amino acids. This is now another analogue for hGM-CSF which may be helpful in further defining regions of this molecule important for activity with human hematopoietic cells. The restricted GM-CSF activity between species which had been previously described
35 seems less evident between canine and human forms.

When caGM-CSF was administered to normal dogs, there were increases in peripheral neutrophil, monocyte, and lymphocyte counts with increased marrow

cellularity. The change in eosinophil counts was more variable. Thrombocytopenia developed in all dogs and appeared to be dose-dependent. Normal to increased numbers of megakaryocytes in the marrow and decreased platelet survival times implicated increased consumption as the mechanism. Whether
5 production of platelets is affected is harder to determine, but the large platelets in the peripheral blood and the rapid rise in platelet counts after stopping caGM-CSF suggest some increased production. Increased production might be expected from direct or indirect stimulation of megakaryocytes by caGM-CSF or as a physiologic response to the thrombocytopenia. Thrombocytopenia also
10 developed in dogs receiving hGM-CSF, and increased consumption was demonstrated as well by determining platelet survival times [38].

The *in vivo* effect of GM-CSF on megakaryocytes and platelets is not well delineated. Platelet counts varied in different animal studies with no changes observed in mice treated with mGM-CSF [50] and platelets noted as unchanged,
15 decreased or increased in primates treated with hGM-CSF [25,51,53] (Farese, A.). Thrombocytopenia has been reported as a complication in several clinical studies of patients with cancer or acquired immunodeficiency syndrome receiving hGM-CSF [52-54]. The possibility of exacerbation of an otherwise stable or subclinical autoimmune thrombocytopenia was considered for some patients. It
20 has also been reported in patients with aplastic anemia that platelet counts 1 hour after platelet transfusion were lower, while on treatment with hGM-CSF, were comparable to pre-treatment increments [55]. Other studies have shown no significant change, or, in some patients with myelodysplasia, an increase in platelet counts [28,29]. There are reports of more rapid recovery of platelet
25 count after autologous marrow transplantation [26,32].

Following peripheral blood counts to assess the *in vivo* effect of GM-CSF on megakaryocytes and platelets is problematic since the platelet count is the result of a dynamic equilibrium between production and consumption of platelets. If GM-CSF increased both production and consumption of platelets, it could yield no
30 change in peripheral counts but some event such as a disease process or treatment could change the equilibrium and increase or decrease counts depending on how production or consumption is affected. Studies on production and utilization of platelets will be necessary to assess the *in vivo* effects of GM-CSF. Assessment of the thrombocytopenia that develops in normal dogs with no predisposing factors
35 may allow a better biological description of an important side effect observed in some patients receiving hGM-CSF. This model may also be useful because this mechanism, where thrombocytopenia may be induced directly or indirectly by

abnormal amounts of a physiologic cytokine like GM-CSF, may also be operative in some disease processes.

Canine GM-CSF has been cloned and expressed and will be useful in analyzing questions about the *in vivo* biological activities of GM-CSF in this animal model. It may also have therapeutic applications in veterinary practice to lessen hematologic toxicity of chemotherapy or as an adjuvant in treatment of infections. Thrombocytopenia is a reproducible dose-dependent phenomenon in normal dogs receiving the species-specific GM-CSF. The decreased survival of platelets as a result of the *in vivo* administration of GM-CSF may also occur in other species, but other variables such as disease processes or chemotherapy may need to be operative to observe significant changes in platelet counts.

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The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto, without departing from the spirit or scope of the invention as set forth herein.

-25-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Nash, Richard
Storb, Rainer
- (ii) TITLE OF INVENTION: Canine Granulocyte Macrophage
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(A) MEDIUM TYPE: Diskette, 5.25 inch, 360 Kb storage
(B) COMPUTER: NBI 64 csTM
(C) OPERATING SYSTEM: MS DOS 3.1
(D) SOFTWARE: NBI
- 20 (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Not available
(B) FILING DATE: -----
(C) CLASSIFICATION: Not available
- (viii) ATTORNEY/AGENT INFORMATION:
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- 30 (2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```

5           -15           -10           -5
Met Trp Leu Gln Asn Leu Leu Phe Leu Gly Thr Val Val Cys Ser Ile

           1           5           10           15
10 Ser Ala Pro Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser Gln His

           20           25           30
Val Asp Ala Ile Gln Glu Ala Leu Ser Leu Leu Asn Asn Ser Asn Asp

15           35           40           45
Val Thr Ala Val Met Asn Lys Ala Val Lys Val Val Ser Glu Val Phe

           50           55           60
20 Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu Gln Leu Tyr Lys

           65           70           75
Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu Thr Met

           80           85           90           95
25 Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Ser Pro

           100          105          110
Cys Ala Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn Leu Lys

30           115          120          125
Asp Phe Leu Phe Asn Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys

```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 127 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

10      1           5           10           15
      Ala Pro Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser Gln His

           20           25           30
      Val Asp Ala Ile Gln Glu Ala Leu Ser Leu Leu Asn Asn Ser Asn Asp

15      35           40           45
      Val Thr Ala Val Met Asn Lys Ala Val Lys Val Val Ser Glu Val Phe

           50           55           60
      Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu Gln Leu Tyr Lys

20      65           70           75
      Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu Thr Met

           80           85           90           95
      Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Ser Pro

25      100          105          110
      Cys Ala Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn Leu Lys

           115          120          125
      Asp Phe Leu Phe Asn Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys

```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 128 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```

10  -1      1              5              10              15
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                20              25              30
    Val Asp Ala Ile Gln Glu Ala Leu Ser Leu Leu Asn Asn Ser Asn Asp

15              35              40              45
    Val Thr Ala Val Met Asn Lys Ala Val Lys Val Val Ser Glu Val Phe

                50              55              60
    Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu Gln Leu Tyr Lys

20              65              70              75
    Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu Thr Met

                80              85              90              95
    Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Ser Pro

25              100             105             110
    Cys Ala Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn Leu Lys

                115             120             125
    Asp Phe Leu Phe Asn Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys

```

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 11 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

1 5 10
10 Glu Ala Leu Ser Leu Leu Asn Asn Ser Asn Asp

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

20 1 5 10
Thr Met Met Ala Asn His Tyr Lys Gln His

15
Cys Pro Pro Thr Pro Glu Ser

25 (2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 809 bases
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (complementary)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

	-15	-10	-5	
	Met Trp Leu Gln Asn Leu Leu Phe Leu Gly Thr Val Val Cys Ser			
	AGGAGG ATG TGG CTG CAG AAC CTG CTT TTC TTG GGC ACT GTG GTC TGC AGC			51
5	1	5	10	
	Ile Ser Ala Pro Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser Gln			
	ATC TCT GCA CCC ACC CGC TCA CCC ACC CTT GTC ACT CGG CCC TCT CAG			99
	15	20	25	30
10	His Val Asp Ala Ile Gln Glu Ala Leu Ser Leu Leu Asn Asn Ser Asn			
	CAC GTG GAT GCC ATC CAG GAA GCC CTG AGC CTT TTG AAC AAC AGT AAT			147
	35	40	45	
	Asp Val Thr Ala Val Met Asn Lys Ala Val Lys Val Val Ser Glu Val			
15	GAC GTG ACT GCT GTG ATG AAT AAA GCA GTA AAA GTG GTC TCT GAA GTG			195
	50	55	60	
	Phe Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu Gln Leu Tyr			
	TTT GAC CCT GAG GGG CCA ACA TGC CTG GAG ACC CGC CTA CAG CTG TAC			243
20	65	70	75	
	Lys Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu Thr			
	AAG GAG GGC CTG CAG GGC AGC CTC ACC AGC CTC AAG AAT CCC TTA ACC			191
	80	85	90	
25	Met Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Ser			
	ATG ATG GCC AAT CAC TAT AAG CAG CAC TGT CCC CCT ACC CCG GAA TCT			339
	95	100	105	110
	Pro Cys Ala Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn Leu			
30	CCC TGT GCA ACC CAG AAT ATT AAC TTC AAA AGT TTC AAA GAG AAC CTG			387

	115	120	125	
	Lys Asp Phe Leu Phe Asn Ile Pro Phe Asp Cys Trp Lys Pro Val Lys			
	AAG GAT TTT CTG TTT AAC ATC CCC TTT GAC TGC TGG AAA CCA GTC AAG			435
5	Lys			
	AAG TGAGGCAGAC CAGTCCAGCC AGGAGCCAGC CCAGTCCAGC CAGAAGCCAG			488
	CCCTGAGAGC ATACCTCATA CCTCACAAGT CACTGCCTTT CTACCCATGG ATTGCTGAAA			548
10	CTCAGGATCT TCACCTTTGA GGGACACCGG GTGGACCAGG GCAGTAGAGG GGGCATGGAC			608
	TTGCTCTGGC CATGCTGCCC GGATACCAGC TTGGTATGGG GAGCGGGGAA TGTTTTATAC			668
	TGGCAGGGAT CAGTAATATT TATTTATATA TTTATGTATT TTAATATTTA TTTATTTATT			728
15	TATTTAAGAT CATACTCTGT ATTTATTCAA GACATTTTAC TATTATAATA AATTATTA			788
	AGCCTGTAA AAAAAAAAAA A			804

(2) INFORMATION FOR SEQ ID NO: 7:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 752 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25 (xi) SEQUENCE DESCRIPTION: SEG ID NO: 7:

1	5	10	
Ala Pro Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser Gln			
GCA CCC ACC CGC TCA CCC ACC CTT GTC ACT CGG CCC TCT CAG .			42

Lys

AAG TGAGGCAGAC CAGTCCAGCC AGGAGCCAGC CCAGTCCAGC CAGAAGCCAG 431

30 CCCTGAGAGC ATACCTCATA CCTCACAAGT CACTGCCTTT CTACCCATGG ATTGCTGAAA 491

CTCAGGATCT TCACCTTTGA GGGACACCGG GTGGACCAGG GCAGTAGAGG GGGCATGGAC 551

TTGCTCTGGC CATGCTGCCC GGATACCAGC TTGGTATGGG GAGCGGGGAA TGTTTTATAC 611

TGGCAGGGAT CAGTAATATT TATTTATATA TTTATGTATT TTAATATTTA TTTATTTATT 671

TATTTAAGAT CATACTCTGT ATTTATTCAA GACATTTTAC TATTATAATA AATTATTAAT 731

5 AGCCTGTTAA AAAAAAAAAA A 752

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated proteinaceous molecule having at least about 90% identity to Amino Acid Sequence I (SEQ ID NO:1), Amino Acid Sequence II (SEQ ID NO:2), or Amino Acid Sequence III (SEQ ID NO:3), or fragments thereof, wherein said molecule or fragments thereof either have an activity of canine granulocyte macrophage colony stimulating factor or are processed *in vivo* to produce a protein having said activity.
2. The isolated proteinaceous molecule according to Claim 1, which comprises Amino Acid Sequence I (SEQ ID NO:1) or fragments thereof.
3. The isolated proteinaceous molecule according to Claim 1, which comprises Amino Acid Sequence II (SEQ ID NO:2) or fragments thereof.
4. The isolated proteinaceous molecule according to Claim 1, which comprises Amino Acid Sequence III (SEQ ID NO:3) or fragments thereof.
5. An isolated proteinaceous molecule which comprises Amino Acid Sequence IV (SEQ ID NO:4) and Amino Acid Sequence V (SEQ ID NO:5) wherein said molecule either has an activity of canine granulocyte macrophage colony stimulating factor or is processed *in vivo* to produce a protein having said activity.
6. An isolated proteinaceous molecule, or one which is substantially identical to said proteinaceous molecule, comprising the mature protein encoded by the nucleic acid sequence as set forth in SEQ ID No: 6.
7. An isolated proteinaceous molecule, or one which is substantially identical to said proteinaceous molecule, comprising the full-length protein encoded by the nucleic acid sequence as set forth in SEQ ID No: 6.

8. The isolated proteinaceous molecule according to any one of Claims 1 - 7 which is attached to at least one glycosyl residue.
9. An isolated polynucleotide, which encodes a proteinaceous molecule having at least about 90% identity to Amino Acid Sequence I (SEQ ID NO:1), Amino Acid Sequence II (SEQ ID NO:2), or Amino Acid Sequence III (SEQ ID NO:3), or fragments thereof, wherein said molecule or fragments thereof either have an activity of canine granulocyte macrophage colony stimulating factor or are processed *in vivo* to produce a protein having said activity.
10. The isolated polynucleotide according to Claim 9, wherein said polynucleotide encodes Amino Acid Sequence I (SEQ ID NO:1) or fragments thereof.
11. The isolated polynucleotide according to Claim 9, wherein said polynucleotide encodes Amino Acid Sequence II (SEQ ID NO:2) or fragments thereof.
12. The isolated polynucleotide according to Claim 9, wherein said polynucleotide encodes Amino Acid Sequence III (SEQ ID NO:3) or fragments thereof.
13. An isolated polynucleotide, which encodes a proteinaceous molecule which comprises Amino Acid Sequence IV (SEQ ID NO:4) and Amino Acid Sequence V (SEQ ID NO:5) wherein said molecule either has an activity of canine granulocyte macrophage colony stimulating factor or is processed *in vivo* to produce a protein having said activity.
14. An isolated polynucleotide which comprises a nucleotide sequence having at least about 90% identity to nucleotide sequence I (SEQ ID NO:6), which encodes a proteinaceous molecule, wherein said molecule has an activity of canine granulocyte macrophage colony stimulating factor or is processed *in vivo* to produce a protein having said activity.
15. An isolated polynucleotide which comprises nucleotide sequence I (SEQ ID NO:6).

16. An isolated polynucleotide which comprises a nucleotide sequence having at least about 90% identity to nucleotide sequence II (SEQ ID NO:7), which encodes a proteinaceous molecule, wherein said molecule has an activity of canine granulocyte macrophage colony stimulating factor or is processed *in vivo* to produce a protein having said activity.
17. An isolated polynucleotide which comprises nucleotide sequence II (SEQ ID NO:7).
18. An isolated polynucleotide encoding a polypeptide comprising the mature protein encoded by the nucleic acid sequence as set forth in SEQ ID No: 6.
19. An isolated polynucleotide encoding a polypeptide comprising the full-length protein encoded by the cDNA insert contained in SEQ ID No: 6.
20. The isolated polynucleotide according to any one of Claims 9 – 19 which is operably linked to a control sequence.
21. A pCMV-CAGM plasmid as deposited with the ATCC and assigned the accession number 20921.
22. A vector comprising the polynucleotide according to any one of Claims 9 –20 or the pCMV-CAGM plasmid according to Claim 21.
23. A host cell transformed or transfected with the vector according to Claim 22.
24. A host cell transformed or transfected with the polynucleotide according to any one of Claims 9-20 or the pCMV-CAGM plasmid according to Claim 21.
25. The cell according to Claim 23 or 24, wherein said cell is prokaryotic.
26. The cell according to Claim 23 or 24, wherein said cell is eukaryotic.

27. The cell according to Claim 26, wherein said cell is a yeast cell.
28. The cell according to Claim 26, wherein said cell is mammalian.
29. The cell according to Claim 28, which is a COS cell.
30. A method of producing a proteinaceous molecule having at least about 90% identity to Amino Acid Sequence I (SEQ ID NO:1), Amino Acid Sequence II (SEQ ID NO:2), or Amino Acid Sequence III (SEQ ID NO:3), or fragments thereof, wherein said molecule or fragments thereof either have an activity of canine granulocyte macrophage colony stimulating factor or are processed *in vivo* to produce a protein having said activity, said method comprising culturing a cell according to any one of Claims 23-29 for a time sufficient to produce said proteinaceous molecule.
31. Use of a therapeutically effective amount of the proteinaceous molecule according to any one of Claims 1 - 8 formulated for administration to an animal to stimulate the formation of colonies of granulocytes or macrophages.
32. The use according to Claim 31, wherein said animal is a dog.
33. Use of the proteinaceous molecule according to any one of Claims 1 – 8 in the manufacture of a medicament for stimulation of the formation of colonies of granulocytes or macrophages.
34. Use of the polynucleotide according to any one of Claims 9-20 or the pCMV-CAGM plasmid according to Claim 21, in the manufacture of a medicament for stimulation of the formation of colonies of granulocytes or macrophages.
35. Use of the vector according to Claim 22 in the manufacture of a medicament for stimulation of the formation of colonies of granulocytes or macrophages.

36. A composition comprising the proteinaceous molecule according to any one of Claims 1- 8 in combination with a pharmaceutically acceptable carrier material.
37. A composition comprising the polynucleotide according to any one of Claims 9-20 or the pCMV-CAGM plasmid according to Claim 21, in combination with a pharmaceutically acceptable carrier material.
38. A composition comprising the vector according to Claim 22 in combination with a pharmaceutically acceptable carrier material.

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-15 -10 -5

Met Trp Leu Gln Asn Leu Leu Phe Leu Gly Thr Val Val Cys Ser Ile

1 5 10 15

Ser Ala Pro Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser Gln His

20 25 30

Val Asp Ala Ile Gln Glu Ala Leu Ser Leu Leu Asn Asn Ser Asn Asp

35 40 45

Val Thr Ala Val Met Asn Lys Ala Val Lys Val Val Ser Glu Val Phe

50 55 60

Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu Gln Leu Tyr Lys

65 70 75

Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu Thr Met

80 85 90 95

Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Ser Pro

100 105 110

Cys Ala Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn Leu Lys

115 120 125

Asp Phe Leu Phe Asn Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys

FIGURE 1A

McCARTHY DEFENDANT
AGENTS FOR THE APPLICANT

1	5	10	15
Ala	Pro	Thr	Arg
Ser	Pro	Thr	Leu
Val	Thr	Arg	Pro
Ser	Gln	His	
20	25	30	
Val	Asp	Ala	Ile
Gln	Glu	Ala	Leu
Ser	Leu	Leu	Asn
Asn	Ser	Asn	Asp
35	40	45	
Val	Thr	Ala	Val
Met	Asn	Lys	Ala
Val	Lys	Val	Val
Ser	Glu	Val	Phe
50	55	60	
Asp	Pro	Glu	Gly
Pro	Thr	Cys	Leu
Glu	Thr	Arg	Leu
Gln	Leu	Tyr	Lys
65	70	75	
Glu	Gly	Leu	Gln
Gly	Ser	Leu	Thr
Ser	Leu	Lys	Asn
Pro	Leu	Thr	Met
80	85	90	95
Met	Ala	Asn	His
Tyr	Lys	Gln	His
Cys	Pro	Pro	Thr
Pro	Glu	Ser	Pro
100	105	110	
Cys	Ala	Thr	Gln
Asn	Ile	Asn	Phe
Lys	Ser	Phe	Lys
Glu	Asn	Leu	Lys
115	120	125	
Asp	Phe	Leu	Phe
Asn	Ile	Pro	Phe
Asp	Cys	Trp	Lys
Pro	Val	Lys	Lys

FIGURE 1B

McCARRELL T. FAULT
AGENTS FOR THE APPLICANT

1 5 10 15
Met Ala Pro Thr Arg Ser Pro Thr Leu Val Thr Arg Pro Ser Gln His
20 25 30
Val Asp Ala Ile Gln Glu Ala Leu Ser Leu Leu Asn Asn Ser Asn Asp
35 40 45
Val Thr Ala Val Met Asn Lys Ala Val Lys Val Val Ser Glu Val Phe
50 55 60
Asp Pro Glu Gly Pro Thr Cys Leu Glu Thr Arg Leu Gln Leu Tyr Lys
65 70 75
Glu Gly Leu Gln Gly Ser Leu Thr Ser Leu Lys Asn Pro Leu Thr Met
80 85 90 95
Met Ala Asn His Tyr Lys Gln His Cys Pro Pro Thr Pro Glu Ser Pro
100 105 110
Cys Ala Thr Gln Asn Ile Asn Phe Lys Ser Phe Lys Glu Asn Leu Lys
115 120 125
Asp Phe Leu Phe Asn Ile Pro Phe Asp Cys Trp Lys Pro Val Lys Lys

FIGURE 1C

M. CARNEY TETRAULT
AGENTS FOR THE APPLICANT

[illegible]

FIGURE 2A

CARTHY TETRAULT
SOLICITOR FOR THE APPLICANT

1	5	10											
Ala	Pro	Thr	Arg	Ser	Pro	Thr	Leu	Val	Thr	Arg	Pro	Ser	Gln
GCA	CCC	ACC	CGC	TCA	CCC	ACC	CTT	GTC	ACT	CGG	CCC	TCT	CAG

15	20	25	30												
His	Val	Asp	Ala	Ile	Gln	Glu	Ala	Leu	Ser	Leu	Leu	Asn	Asn	Ser	Asn
CAC	GTG	GAT	GCC	ATC	CAG	GAA	GCC	CTG	AGC	CTT	TTG	AAC	AAC	AGT	AAT

35	40	45													
Asp	Val	Thr	Ala	Val	Met	Asn	Lys	Ala	Val	Lys	Val	Val	Ser	Glu	Val
GAC	GTG	ACT	GCT	GTG	ATG	AAT	AAA	GCA	GTA	AAA	GTG	GTC	TCT	GAA	GTG

50	55	60													
Phe	Asp	Pro	Glu	Gly	Pro	Thr	Cys	Leu	Glu	Thr	Arg	Leu	Gln	Leu	Tyr
TTT	GAC	CCT	GAG	GGG	CCA	ACA	TGC	CTG	GAG	ACC	CGC	CTA	CAG	CTG	TAC

65	70	75													
Lys	Glu	Gly	Leu	Gln	Gly	Ser	Leu	Thr	Ser	Leu	Lys	Asn	Pro	Leu	Thr
AAG	GAG	GGC	CTG	CAG	GGC	AGC	CTC	ACC	AGC	CTC	AAG	AAT	CCC	TTA	ACC

80	85	90													
Met	Met	Ala	Asn	His	Tyr	Lys	Gln	His	Cys	Pro	Pro	Thr	Pro	Glu	Ser
ATG	ATG	GCC	AAT	CAC	TAT	AAG	CAG	CAC	TGT	CCC	CCT	ACC	CCG	GAA	TCT

95	100	105	110												
Pro	Cys	Ala	Thr	Gln	Asn	Ile	Asn	Phe	Lys	Ser	Phe	Lys	Glu	Asn	Leu
CCC	TGT	GCA	ACC	CAG	AAT	ATT	AAC	TTC	AAA	AGT	TTC	AAA	GAG	AAC	CTG

115	120	125													
Lys	Asp	Phe	Leu	Phe	Asn	Ile	Pro	Phe	Asp	Cys	Trp	Lys	Pro	Val	Lys
AAG	GAT	TTT	CTG	TTT	AAC	ATC	CCC	TTT	GAC	TGC	TGG	AAA	CCA	GTC	AAG

Lys
AAG TGAGGCAGAC CAGTCCAGCC AGGAGCCAGC CCAGTCCAGC CAGAAGCCAG

CCCTGAGAGC ATACCTCATA CCTCACAAGT CACTGCCTTT CTACCCATGG ATTGCTGAAA

CTCAGGATCT TCACCTTTGA GGGACACCGG GTGGACCAGG GCAGTAGAGG GGGCATGGAC

TTGCTCTGGC CATGCTGCCC GGATACCAGC TTGGTATGGG GAGCGGGGAA TGTTTTATAC

TGGCAGGGAT CAGTAATATT TATTTATATA TTTATGTATT TTAATATTTA TTTATTTATT

TATTTAAGAT CATACTCTGT ATTTATTCAA GACATTTTAC TATTATAATA AATTATTAAA

AGCCTGTAA AAAAAAAAAA A

FIGURE 2B

CARINA TETPAULT
AGENTS FOR THE APPLICANT

PCR to Construct Full-Length cDNA

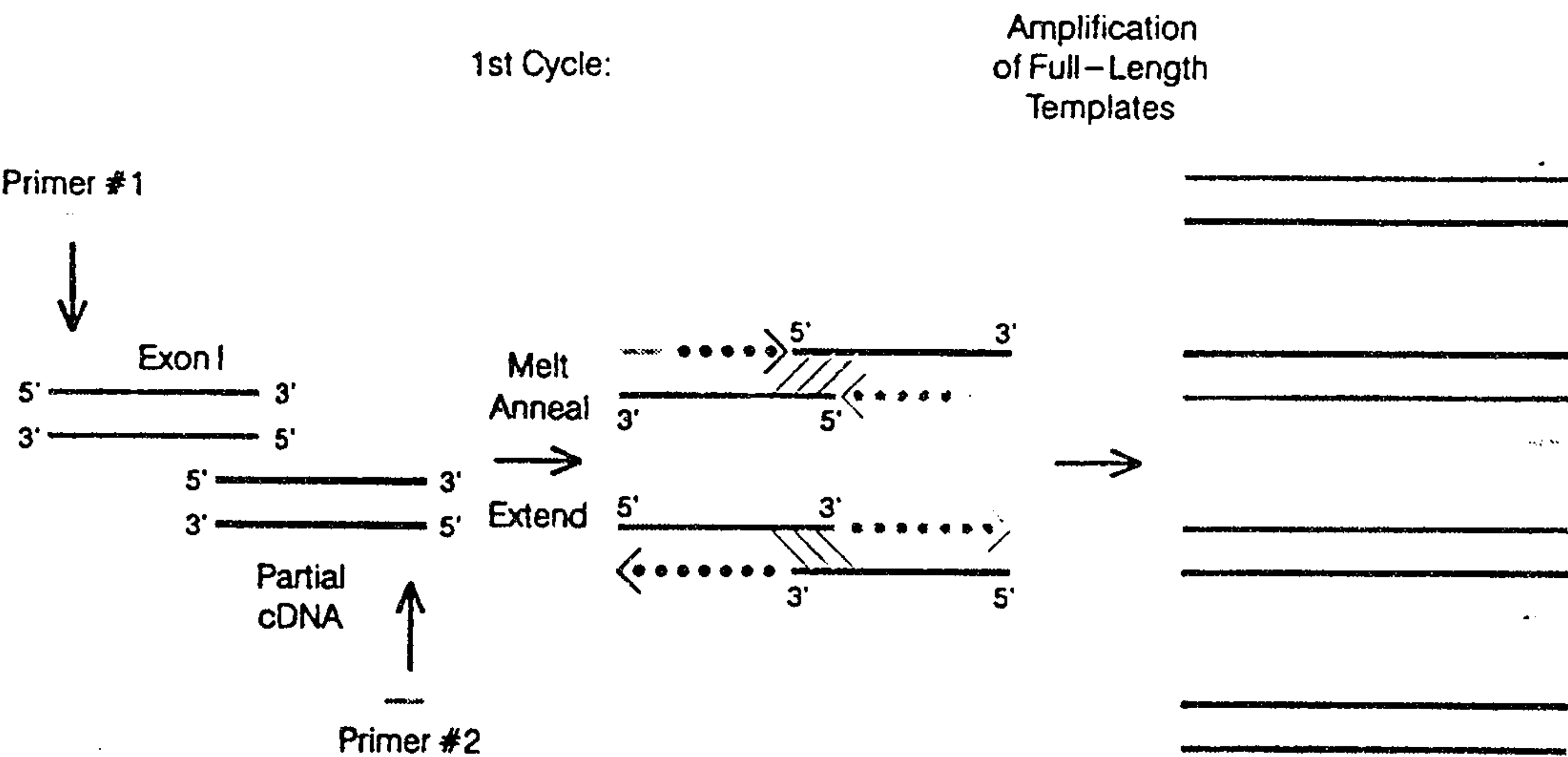


FIGURE 3

ATG	TGG	CTG	CAG	AAC	CTG	CTT	TTC	TTG	GGC	ACT	GTG	GTC	TGC	AGC	51
M	W	L	Q	N	L	L	F	L	G	T	V	V	C	S	
ATC	TCT	GCA	CCC	ACC	CGC	TCA	CCC	ACC	CTT	GTC	ACT	CGG	CCC	TCT	96
I	S	A	P	T	R	S	P	T	L	V	T	R	P	S	
CAG	CAC	GTG	GAT	GCC	ATC	CAG	GAA	GCC	CTG	AGC	CTT	TTG	AAC	AAC	141
Q	H	V	D	A	I	Q	E	A	L	S	L	L	N	N	
AGT	AAT	GAC	GTG	ACT	GCT	GTG	ATG	AAT	AAA	GCA	GTA	AAA	GTG	GTC	186
S	N	D	V	T	A	V	M	N	K	A	V	K	V	V	
TCT	GAA	GTG	TTT	GAC	CCT	GAG	GGG	CCA	ACA	TGC	CTG	GAG	ACC	CGC	231
S	E	V	F	D	P	E	G	P	T	C	L	E	T	R	
CTA	CAG	CTG	TAC	AAG	GAG	GGC	CTG	CAG	GGC	AGC	CTC	ACC	AGC	CTC	276
L	Q	L	Y	K	E	G	L	Q	G	S	L	T	S	L	
AAG	AAT	CCC	TTA	ACC	ATG	ATG	GCC	AAT	CAC	TAT	AAG	CAG	CAC	TGT	321
K	N	P	L	T	M	M	A	N	H	Y	K	Q	H	C	
CCC	CCT	ACC	CCG	GAA	TCT	CCC	TGT	GCA	ACC	CAG	AAT	ATT	AAC	TTC	366
P	P	T	P	E	S	P	C	A	T	Q	N	I	N	F	
AAA	AGT	TTC	AAA	GAG	AAC	CTG	AAG	GAT	TTT	CTG	TTT	AAC	ATC	CCC	411
K	S	F	K	E	N	L	K	D	F	L	F	N	I	P	
TTT	GAC	TGC	TGG	AAA	CCA	GTC	AAG	AAG	TGA						438
F	D	C	W	K	P	V	K	K							

GGCAGACCA GTCCAGCCAG GAGCCAGCCC AGTCCAGCCA
GAAGCCAGCC CTGAGAGCAT ACCTCATACC TCACAAGTCA CTGCCTTTCT ACCCATGGAT
TGCTGAAACT CAGGATCTTC ACCTTTGAGG GACACCGGGT GGACCAGGGC AGTAGAGGGG
GCATGGACTT GCTCTGGCCA TGCTGCCCGG ATACCAGCTT GGTATGGGGA GCGGGGAATG
TTTTATACTG GCAGGGATCA GTAATATTTA TTTATATATT TATGTATTTT AATATTTATT
TATTTATTTA TTTAAGATCA TACTCTGTAT TTATTCAAGA CATTTTACTA TTATAATAAA
TTATTAAAAG CCTGTTAAAA AAAAAAAAAA

FIGURE 4

CARNEY TETRAULT
AGENTS FOR THE APPLICANT

- - identical amino acids as caGM-CSF
- . . gaps to maximize alignment
- ↓ ↓ arrows bracket signal peptide
- * * indicate N-glycosylation site
- ■ cysteine
- underlined functional region

CARNEY TETRAULT
 1015 FOLLY THE APPLICANT

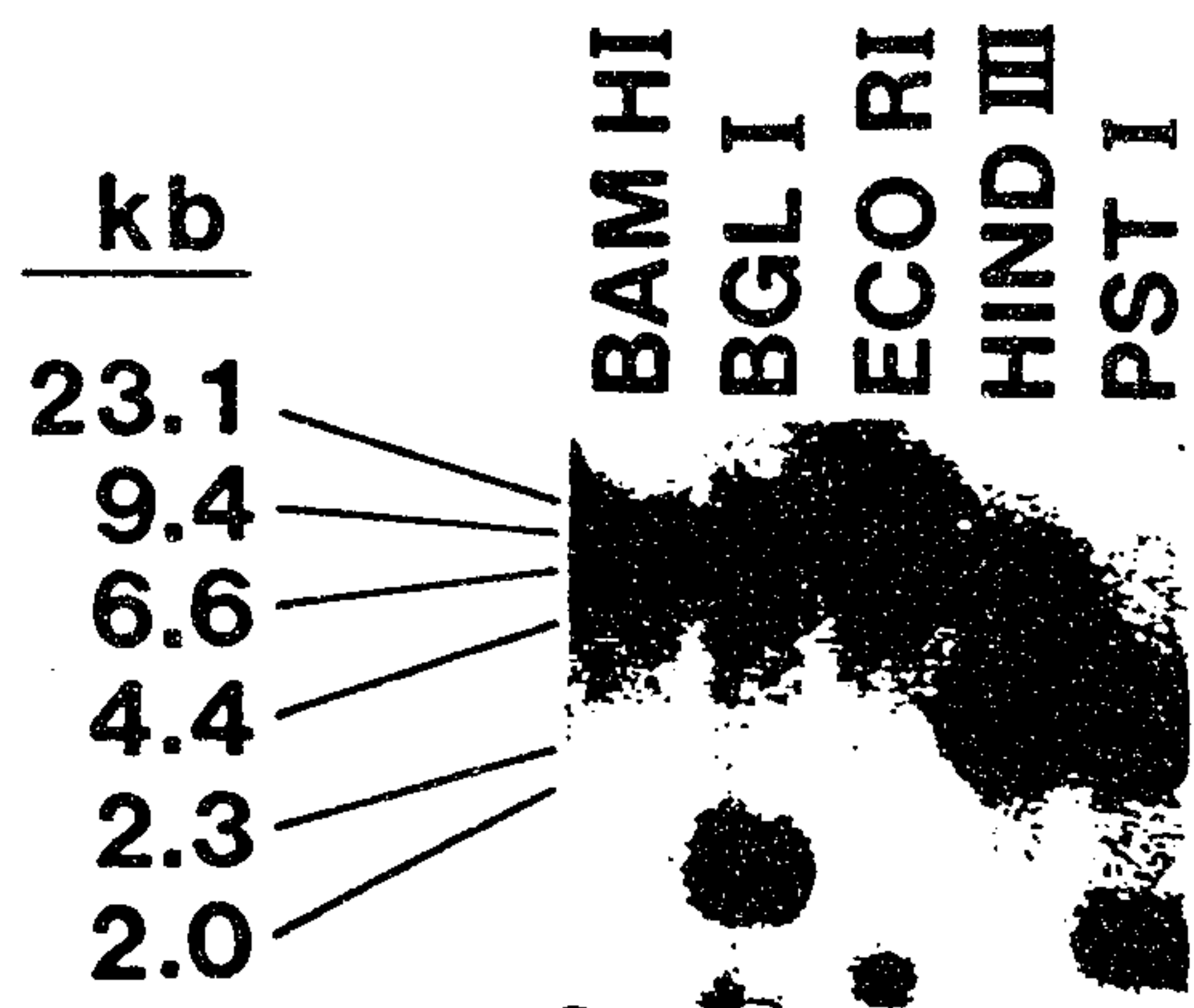


FIGURE 6

W. DARTHY TÉTRAULT
ATTORNEYS FOR THE APPLICANT

Canine CFU – GM Assay

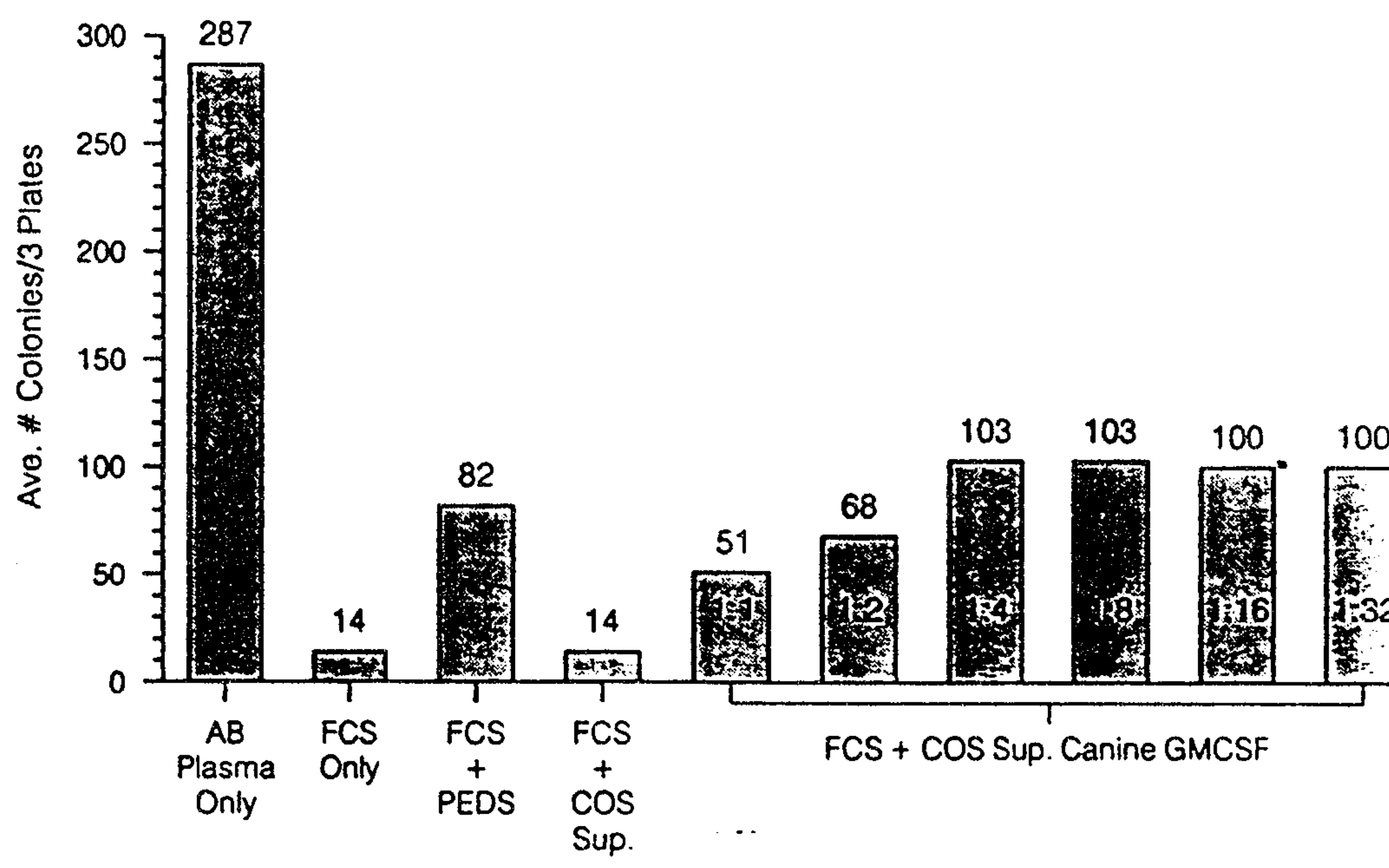


FIGURE 7

LABORATORY REPORT
AGENTS FOR THE APPLICANT

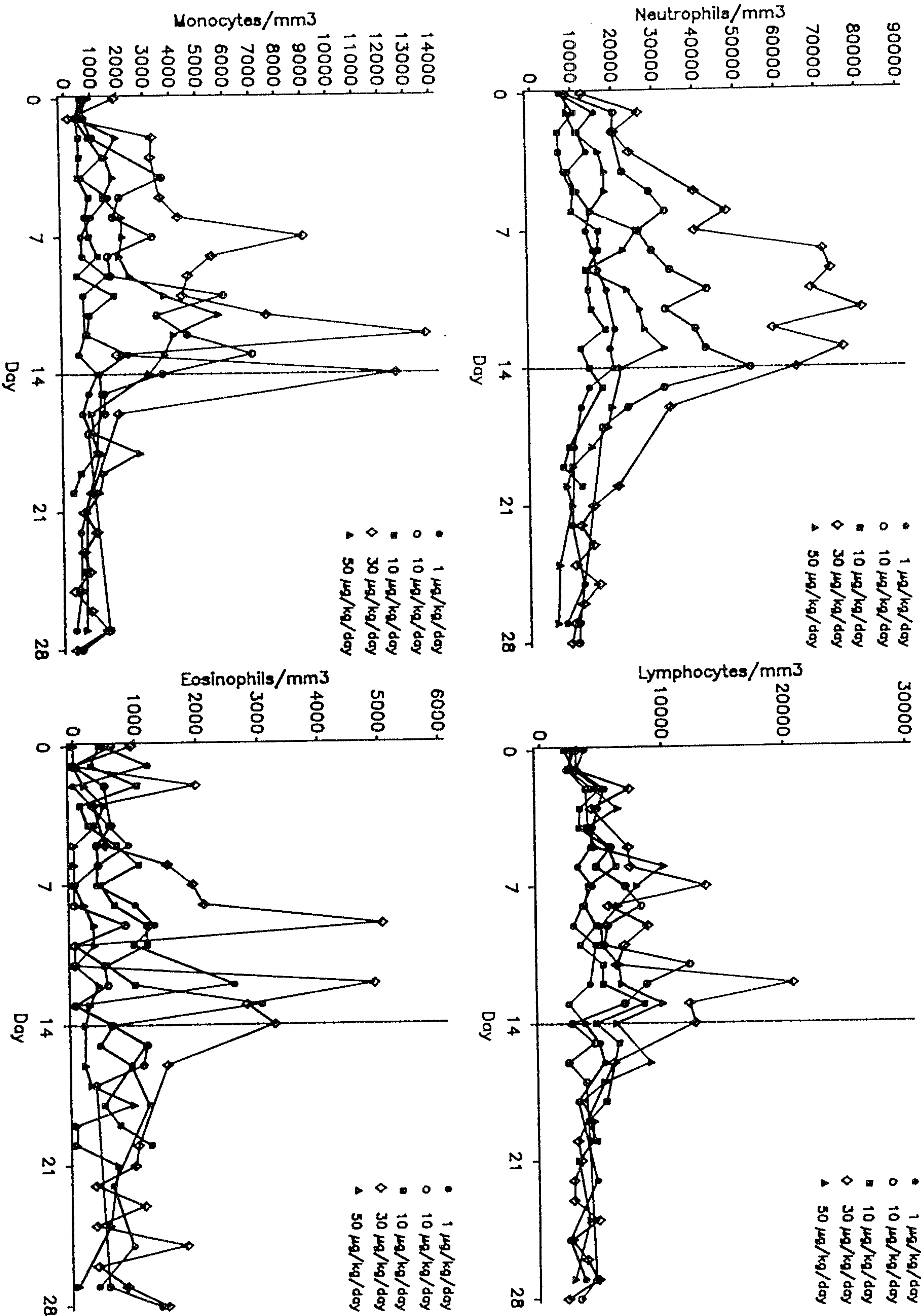


FIGURE 8

ANTHONY TETRAULT
POINTS FOR THE APPLICANT

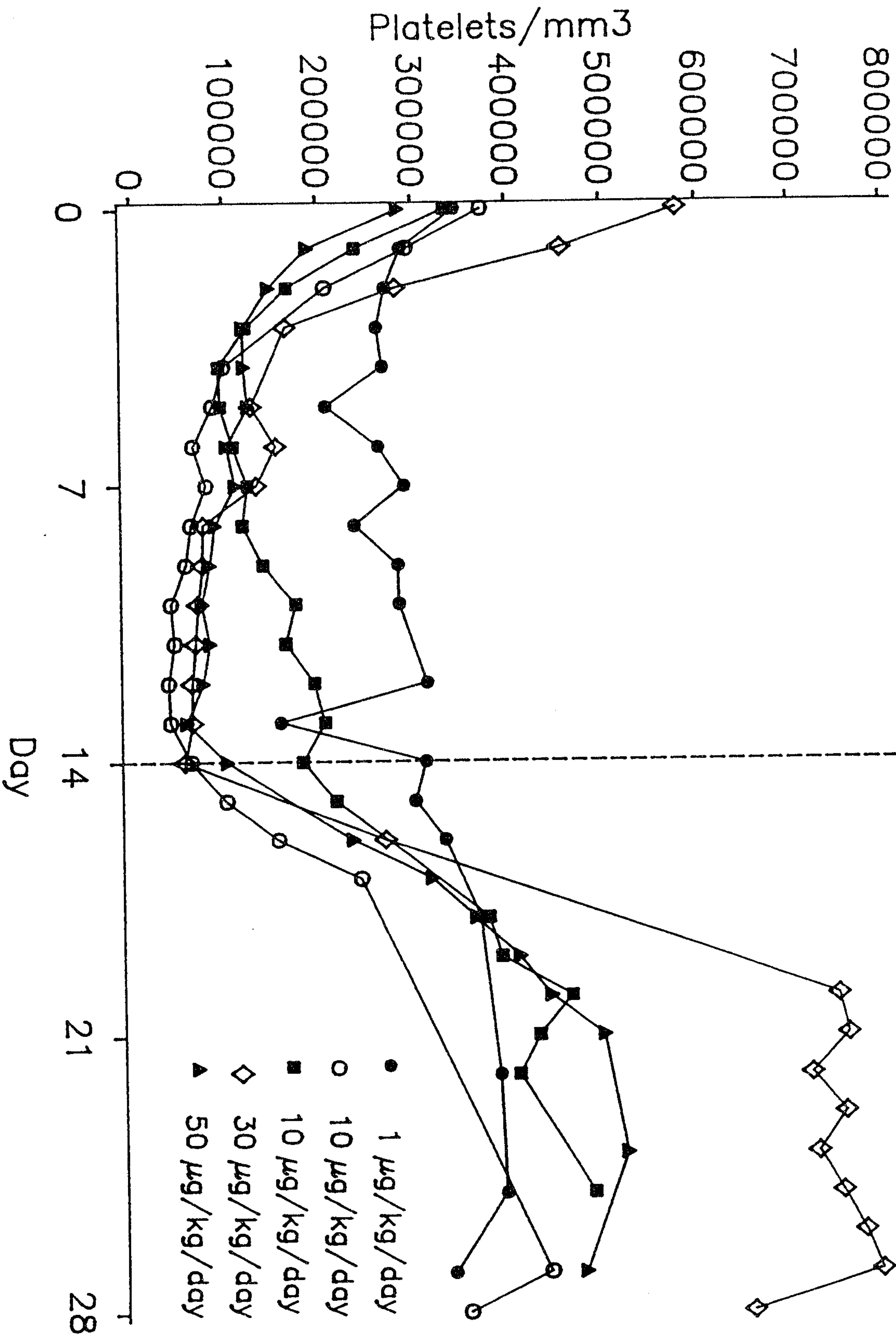


FIGURE 9

SCARATHY TÉTRAULT
POINTS FOR APPLICANT