



Office de la Propriété
Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2167090 C 2002/05/14

(11)(21) **2 167 090**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 1995/03/30
(87) Date publication PCT/PCT Publication Date: 1995/10/12
(45) Date de délivrance/Issue Date: 2002/05/14
(85) Entrée phase nationale/National Entry: 1996/01/11
(86) N° demande PCT/PCT Application No.: US 1995/003776
(87) N° publication PCT/PCT Publication No.: 1995/026746
(30) Priorités/Priorities: 1994/03/31 (08/221,768) US;
1994/05/31 (08/252,628) US; 1994/10/12 (08/321,488) US;
1994/11/30 (08/347,780) US

(51) Cl.Int.⁶/Int.Cl.⁶ C12N 15/18, A61K 47/48, A61K 38/18,
C12N 5/10, C07K 16/22, C12N 1/21, C07K 14/475,
C07K 17/08

(72) Inventeurs/Inventors:
Samal, Babru B., US;
Kinstler, Olaf B., US;
Hunt, Pamela, US;
Bosselman, Robert A., US;
Bogenberger, Jakob M., US;
Bartley, Timothy D., US

(73) Propriétaire/Owner:
AMGEN INC., US

(74) Agent: GOWLING LAFLEUR HENDERSON LLP

(54) Titre : COMPOSITIONS ET METHODES DE STIMULATION DE LA CROISSANCE ET DE LA DIFFERENTIATION
DES MEGACARYOCYTES
(54) Title: COMPOSITIONS AND METHODS FOR STIMULATING MEGAKARYOCYTE GROWTH AND
DIFFERENTIATION

(57) **Abrégé/Abstract:**

Disclosed are novel proteins, referred to as megakaryocyte growth and development factors (MGDFs; also generally referred to as Mpl ligands), that have a biological activity of stimulating the growth of megakaryocytes and augmenting the differentiation or maturation of megakaryocytes, ultimately to result in the production of platelets. MGDF derivatives comprising MGDF molecules attached to water-soluble polymers, such as polyethylene glycol, are also disclosed, along with methods for their preparation. Also disclosed are processes for obtaining the MGDFs in homogeneous form from natural sources and producing them by recombinant genetic engineering techniques from mammals, including humans.





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/16, 38/18, C07H 21/04, C07K 1/12, 14/00, 14/435, 14/475, C12N 15/00, 15/09, 15/12	A1	(11) International Publication Number: WO 95/26746 (43) International Publication Date: 12 October 1995 (12.10.95)
(21) International Application Number: PCT/US95/03776 (22) International Filing Date: 30 March 1995 (30.03.95) (30) Priority Data: 08/221,768 31 March 1994 (31.03.94) US 08/252,628 31 May 1994 (31.05.94) US 08/321,488 12 October 1994 (12.10.94) US 08/347,780 30 November 1994 (30.11.94) US (71) Applicant (for all designated States except US): AMGEN INC. [US/US]; Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): BARTLEY, Timothy, D. [US/US]; 2431 McCrea Road, Thousand Oaks, CA 91362 (US). BOGENBERGER, Jakob, M. [US/US]; 2242 Barbara Drive, Camarillo, CA 93010 (US). BOSSELMAN, Robert, A. [US/US]; 3301 Baccarat Street, Thousand Oaks, CA 91362 (US). HUNT, Pamela [US/US]; 2431 McCrea Road, Thousand Oaks, CA 91362 (US). KINSTLER, Olaf, B. [GB/US]; 533 North Oaktree Unit A, Thousand Oaks, CA 91360 (US). SAMAL, Babru, B. [US/US]; 1136 Broadview Drive, Moorpark, CA 93021 (US).		(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US). (81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published With international search report. 2167090
(54) Title: COMPOSITIONS AND METHODS FOR STIMULATING MEGAKARYOCYTE GROWTH AND DIFFERENTIATION (57) Abstract Disclosed are novel proteins, referred to as megakaryocyte growth and development factors (MGDFs; also generally referred to as Mpl ligands), that have a biological activity of stimulating the growth of megakaryocytes and augmenting the differentiation or maturation of megakaryocytes, ultimately to result in the production of platelets. MGDF derivatives comprising MGDF molecules attached to water-soluble polymers, such as polyethylene glycol, are also disclosed, along with methods for their preparation. Also disclosed are processes for obtaining the MGDFs in homogeneous form from natural sources and producing them by recombinant genetic engineering techniques from mammals, including humans.		

-1-

COMPOSITIONS AND METHODS FOR STIMULATING
MEGAKARYOCYTE GROWTH AND DIFFERENTIATION

5

Field of the Invention

The present invention relates to novel proteins, referred to herein synonymously as Mpl ligands or MGDFs, that stimulate the growth of megakaryocytes and augment the differentiation or maturation of megakaryocytes, with the ultimate effect of increasing the numbers of platelets. Also provided are processes for obtaining the proteins in homogeneous form from natural sources and producing them by recombinant genetic engineering techniques.

In another aspect, the present invention broadly relates to a novel class of MGDF derivatives wherein an MGDF molecule is attached to a water soluble polymer, and methods for preparing such molecules. In yet another aspect, the present invention relates to MGDF derivatives wherein an MGDF molecule is attached to one or more polyethylene glycol ("PEG") groups, and methods of their preparation.

25

Background of the Invention

At least two broad areas of research are involved in the present invention. The first relates to the development of megakaryocytes and subsequent production of platelets, and the second relates to a polypeptide member of a growth factor receptor family, referred to herein as the Mpl receptor, and ligands thereof. Each of these areas of research will now be outlined in the following.

35

2167090

-2-

A. Platelet Production from Megakaryocytes

Blood platelets are circulating cells that are crucial for the prevention of bleeding and for blood coagulation. Megakaryocytes are the cellular source of platelets and arise from a common bone marrow precursor cell which gives rise to all hematopoietic cell lineages. This common precursor cell is known as the pluripotent stem cell or PPSC.

A hierarchy of megakaryocytic progenitor cells has been defined based on the time of appearance and size of megakaryocyte (MK) colonies appearing in *in vitro* culture systems in response to appropriate growth factors. The burst-forming unit megakaryocyte (BFU-MK) is the most primitive megakaryocyte progenitor cell. BFU-MK are thought ultimately to produce numerous colony forming unit megakaryocytes (CFU-MK), which are more differentiated MK progenitor cells.

As the MK cells undergo subsequent differentiation, they lose the ability to undergo mitosis but acquire an ability to endoreduplicate. Endoreduplication (or endomitosis) is the phenomenon in cells of nuclear division in the absence of cell division. Endoreduplication ultimately results in an MK which is polyploid. Further MK maturation results in acquisition of cytoplasmic organelles and membrane constituents that characterize platelets.

Platelets are produced from mature MK's by a poorly defined process that has been suggested to be a consequence of MK physical fragmentation, or other mechanisms. Observations of extensive membranous structures within megakaryocytes has led to a model of platelet formation in which a demarcation membrane system outlines nascent platelets within the cell body. Another model of platelet formation has developed from observations that megakaryocytes will form long

-3-

cytoplasmic processes constricted at platelet-sized intervals from which platelets presumably break off due to blood flow pressures in the marrow and/or in the lung. These cytoplasmic processes were termed
5 proplatelets by Becker and DeBruyn to reflect their presumed precursor role in platelet formation. See Becker and DeBruyn, *Amer. J. Anat.* **145**: 183 (1976).

FIG. 1 presents an overview of the various precursor cells involved in megakaryocyte and platelet
10 development. The cell at the far left-hand side of the figure represents a PPSC, and the additional cells to the right of the PPSC in the figure represent BFU-MK, followed by CFU-MK. The cell that is undergoing endoreduplication, which is located immediately to the
15 right of the PPSC in the figure, is a mature megakaryocyte cell. As a result of endomitosis, this cell has become polyploid. The next structure to the right includes long cytoplasmic processes emerging from the polyploid nucleus of the mature megakaryocyte cell.
20 In the far right-hand side of the figure are shown a number of platelets that have been produced by fragmentation of the cytoplasmic processes.

The following is a summary of some prior publications relating to the above description of
25 megakaryocyte maturation and the production of platelets:

1. Williams, N. and Levine, R.F., *British Journal of Haematology* **52**: 173-180 (1982).
30
2. Levin, J., *Molecular Biology and Differentiation of Megakaryocytes*, pub. Wiley-Liss, Inc.: 1-10 (1990).
3. Gewirtz, A.M., *The Biology of Hematopoiesis*, pub.
35 Wiley-Liss, Inc.: 123-132 (1990).

2167090

-4-

4. Han, Z.C., et al., *Int. J. Hematol.* **54**: 3-14
(1991).
5. Nieuwenhuis, H.K. and Sixma, J., *New Eng. J. of*
5 *Med.* **327**: 1812-1813 (1992).
6. Long, M., *Stem Cells* **11**: 33-40 (1993).

10 B. Regulation of Platelet Formation

A large body of data generated in many laboratories indicates that platelet production is regulated by humoral factors. The complexity of this biological process was not originally appreciated and
15 currently it appears that a number of human growth factors possess this capability.

Megakaryocyte regulation occurs at multiple cellular levels. A number of cytokines enhance platelet production by expanding the progenitor cell pool. A
20 second group of humoral growth factors serves as maturation factors acting on more differentiated cells to promote endoreduplication. In addition, there appear to be two independent biofeedback loops regulating these processes.

25 Several lineage nonspecific hematopoietic growth factors exert important effects on MK maturation. Granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3), IL-6, IL-11, leukemia inhibitory factor (LIF), and erythropoietin (EPO) each
30 individually promote human MK maturation *in vitro* as determined by their effects on MK size, number, or ploidy. The MK maturational effects of LIF, IL-6, and IL-11 are either partially (LIF and IL-6) or totally (IL-11) additive to those of IL-3. Such data from these
35 prior publications suggested that combinations of cytokines may be necessary to promote MK maturation *in*

-5-

vivo.

The following is a summary of some prior publications relating to the regulation of megakaryocyte and platelet production:

5

7. Hoffman, R. et al., *Blood Cells* **13**: 75-86 (1987).

8. Murphy, M.J., *Hematology/Oncology Clinics of North America* **3** (3): 465-478 (1988).

10

9. Hoffman, R., *Blood* **74** (4): 1196-1212 (1989).

10. Mazur, E.M. and Cohen, J.L., *Clin. Pharmacol. Ther.*, **46** (3): 250-256 (1989).

15

11. Gewirtz, A.M. and Calabretta, B., *Int. J. Cell Cloning* **8**: 267-276 (1990).

20

12. Williams, N., *Progress in Growth Factor Research* **2**: 81-95 (1990).

13. Gordon, M.S. and Hoffman, R., *Blood* **80** (2): 302-307 (1992).

25

14. Hunt, P. et al., *Exp. Hematol.* **21**: 372-281 (1993).

15. Hunt, P. et al., *Exp. Hematol.* **21**: 1295-1304 (1993).

30

It has also been reported (see reference 16) that human aplastic serum contains a megakaryocyte colony stimulating activity distinct from IL-3, granulocyte colony stimulating factor, and factors present in lymphocyte-conditioned medium. However, the molecule responsible for this activity was neither

35 isolated nor characterized in the prior art.

2167090

-6-

16. Mazur, E.M., et al., *Blood* **76**: 290-297 (1990).

5 C. The Mpl Receptor

The myeloproliferative leukemia virus (MPLV) is a murine replication-defective retrovirus that causes acute leukemia in infected mammals. It has been
10 discovered that a gene expressed by MPLV consists of a part of the gene that encodes the retroviral envelope (or external protein coat) of the virus fused to a sequence that is related to the cytokine receptor family, including the receptors for GM-CSF, G-CSF,
15 and EPO.

Expression of the MPLV gene described above has the interesting biological property of causing murine progenitor cells of various types to immediately acquire growth factor independence for both
20 proliferation and terminal maturation. Moreover, some cultures of bone marrow cells acutely transformed by MPLV contained megakaryocytes, suggesting a connection between the MPLV gene and megakaryocyte growth and differentiation.

25 It is now recognized that the MPLV viral gene (referred to as v-Mpl) has a homolog in mammalian cells, which is referred to as a cellular Mpl gene (or c-Mpl). Using v-Mpl-derived probes, a cDNA corresponding to the human c-Mpl gene was cloned. See PCT published
30 application WO 92/07074 (published April 30, 1992; discussed below). Sequence analysis has shown that the protein encoded by the c-Mpl gene product belongs to the highly conserved cytokine receptor superfamily, just like the homologous v-Mpl gene product.

35 This cellular gene, c-Mpl, is thought to play a functional rôle in hematopoiesis based on the

-7-

observation that its expression was found in bone marrow, spleen, and fetal liver from normal mice by RNase probe protection and RT-PCR experiments, but not in other tissues. In particular, c-Mpl is expressed on megakaryocytes. It has also been demonstrated that the human cellular gene, human c-Mpl, is expressed in CD34 positive cells, including purified megakaryocytes and platelets. CD34 is an antigen that is indicative of early hematopoietic progenitor cells. Furthermore, exposure of CD34 positive cells to synthetic oligodeoxynucleotides that are anti-sense to the c-Mpl mRNA or message significantly inhibits the colony forming ability of CFU-MK megakaryocyte progenitors, but has no effect on erythroid or granulomacrophage progenitors.

The above data and observations suggest that c-Mpl encodes a cell surface molecule, referred to herein as the Mpl receptor, which binds to a ligand, that activates the receptor, possibly leading to production and/or development of megakaryocytes.

PCT patent publication WO 92/07074 is directed to the sequence of the protein produced by the c-Mpl gene, from both human and murine sources. This gene product, which is thought to be a receptor as explained above, is made up of at least three general regions or domains: an extracellular domain, a transmembrane domain, and an intracellular (or cytoplasmic) domain. Attached together, these domains make up the intact Mpl receptor. This PCT publication also refers to a soluble form of the receptor that substantially corresponds to the extracellular domain of the mature c-Mpl protein. The intracellular domain contains a hydrophobic region that, when attached via the transmembrane region to the extracellular domain of the protein, renders the overall protein subject to aggregation and insolubility. On the other hand, when the extracellular domain of the c-Mpl

2167090

-8-

gene product is separated from the transmembrane domain and the intracellular domain, it becomes soluble, hence the extracellular form of the protein is referred to as a "soluble" form of the receptor.

5 The following is a summary of some prior publications relating to the above description of the v-Mpl and c-Mpl receptors and genes:

- 10 17. Wendling, F., et al., *Leukemia* **3** (7): 475-480 (1989).
18. Wendling, F., et al., *Blood* **73** (5): 1161-1167 (1989).
- 15 19. Souyri, M., et al., *Cell* **63**: 1137-1147 (1990).
20. Vigon, I., et al., *Proc. Natl. Acad. Sci. USA* **89**: 5640-5644 (1992).
- 20 21. Skoda, R.C., et al., *The EMBO Journal* **12** (7): 2645-2653 (1993).
22. Ogawa, M., *Blood* **81** (11): 2844-2853 (1993).
- 25 23. Methia, N., et al., *Blood* **82** (5): 1395-1401 (1993).
24. Wendling, F., et al., *Blood* **80**: 246a (1993).
- 30 D. The need for an agent capable of stimulating platelet production.

 It has been reported recently that platelet transfusions are being administered at an ever
35 increasing rate at medical centers in North America, Western Europe, and Japan. See Gordon, M.S. and

-9-

Hoffman, R., *Blood* **80** (2): 302-307 (1992). This increase appears to be due in large measure to advances in medical technology and greater access to such technologies as cardiac surgery and bone marrow, heart, and liver transplantation. Dose intensification as a means of delivering therapies to cancer patients and the HIV-1 epidemic have also contributed to the heavy demand on the platelet supply.

Platelet usage carries with it the possibility of transmission of the many blood-born infectious diseases as well as alloimmunization. Moreover, the production of purified platelets is an expensive endeavor and hence the increasing use of such platelets increases overall medical costs. As a result, there exists an acute need for new and improved methods for producing platelets for human uses.

Exemplary prior approaches to enhancing platelet production are described in the following:

U.S. patent 5,032,396 reports that interleukin-7 (IL-7) is capable of stimulating platelet production. Interleukin-7 is also known as lymphopoietin-1 and is a lymphopoietic growth factor capable of stimulating growth of B- and T-cell progenitors in bone marrow. Published PCT application serial number 88/03747, filed October 19, 1988 and European patent application number 88309977.2, filed October 24, 1988 disclose DNA's, vectors, and related processes for producing mammalian IL-7 proteins by recombinant DNA technology. The data presented in the U.S. patent show that IL-7 can increase circulating platelets in normal and sublethally irradiated mice.

U.S. patent 5,087,448 discloses that megakaryocytes and platelets can be stimulated to proliferate in mammals by treating them with interleukin-6. Recombinant human interleukin-6 is a 26,000 molecular weight glycoprotein with multiple

2167090

-10-

biological activities. The data presented in this patent show that IL-6 has an effect of increasing colonies of megakaryocytes *in vitro*.

None of the above-cited patents mentions anything with respect to the Mpl ligands that are involved in the present invention.

In spite of the above disclosures, there remains a strong need for new stimulators of megakaryocytes and/or platelets in mammals.

10

E. Background relating to Chemically Modified MGDF

Proteins for therapeutic use are currently available in suitable forms in adequate quantities largely as a result of the advances in recombinant DNA technologies. Chemical derivatives of such proteins may effectively block a proteolytic enzyme from physical contact with the protein backbone itself, and thus prevent degradation. Additional advantages may include, under certain circumstances, increasing the stability and circulation time of the therapeutic protein and decreasing immunogenicity. However, it should be noted that the effect of modification of a particular protein cannot be predicted. A review article describing protein modification and fusion proteins is Francis, *Focus on Growth Factors 3*: 4-10 (May 1992) (published by Mediscript*, Mountview Court, Friern Barnet Lane, London N20, OLD, UK).

Polyethylene glycol ("PEG" or "peg") is one such chemical moiety which has been used in the preparation of therapeutic protein products. For example Adagen®, a formulation of pegylated adenosine deaminase is approved for treating severe combined immunodeficiency disease; pegylated superoxide dismutase has been in clinical trials for treating head injury;

*Trademark

B

-11-

pegylated alpha interferon has been tested in phase I clinical trials for treating hepatitis; pegylated glucocerebrosidase and pegylated hemoglobin are reported to have been in preclinical testing. For some
5 proteins, the attachment of polyethylene glycol has been shown to protect against proteolysis, Sada, et al., J. Fermentation Bioengineering 71: 137-139 (1991), and methods for attachment of certain polyethylene glycol moieties are available. See U.S. Patent No. 4,179,337,
10 Davis et al., "Non-Immunogenic Polypeptides," issued December 18, 1979; and U.S. Patent No. 4,002,531, Royer, "Modifying enzymes with Polyethylene Glycol and Product Produced Thereby," issued January 11, 1977. For a review, see Abuchowski et al., in Enzymes as Drugs.
15 (J.S. Holcerberg and J. Roberts, eds. pp. 367-383 (1981)).

Other water soluble polymers have been used to modify proteins, such as copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose,
20 dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, and poly amino acids (either homopolymers or random copolymers).

For polyethylene glycol, a variety of means
25 have been used to attach the polyethylene glycol molecules to the protein. Generally, polyethylene glycol molecules are connected to the protein via a reactive group found on the protein. Amino groups, such as those on lysine residues or at the N-terminus, are
30 convenient for such attachment. For example, Royer (U.S. Pat. No. 4,002,531, above) states that reductive alkylation was used for attachment of polyethylene glycol molecules to an enzyme. EP 0 539 167, published April 28, 1993, Wright, "Peg Imidates and Protein
35 Derivates Thereof" states that peptides and organic compounds with free amino group(s) are modified with an

B

2167090

-12-

imidate derivative of PEG or related water-soluble organic polymers. U.S. Patent No. 4,904,584, Shaw, issued February 27, 1990, relates to the modification of the number of lysine residues in proteins for the attachment of polyethylene glycol molecules via reactive amine groups.

One specific therapeutic protein which has been chemically modified is granulocyte colony stimulating factor, "G-CSF." See European patent publications EP 0 401 384, EP 0 473, 268, and EP 0 335 423.

Another example is pegylated IL-6, EP 0 442 724, entitled, "Modified hIL-6," which discloses polyethylene glycol molecules added to IL-6. EP 0 154 316, published September 11, 1985, reports reacting a lymphokine with an aldehyde of polyethylene glycol.

The ability to modify MGDF is unknown in the art since the susceptibility of each individual protein to modification is determined by the specific structural parameters of that protein. Moreover, the effect of such a modification on the biological properties of each protein is unpredictable from the art. Because of the many clinical applications of MGDF, as set forth herein, a derivatized MGDF product with altered properties is desirable. Such molecules may have increased half-life and/or activity *in vivo*, as well other properties.

Pegylation of protein molecules will generally result in a mixture of chemically modified protein molecules. As an illustration, protein molecules with five lysine residues and a free amino group at the N-terminus reacted in the above methods may result in a heterogeneous mixture, some having six polyethylene glycol moieties, some five, some four, some three, some two, some one and some zero. And, among the molecules with several, the polyethylene glycol moieties may not

-13-

be attached at the same location on different molecules. It will frequently be desirable to obtain a homogeneous product that contains substantially all one or a small number (e.g., 2-3) of modified protein species that vary
5 in the number and/or location of chemical moieties, such as PEG. Nevertheless, mixtures of, e.g., mono-, di- and/or tri-pegylated species may be desirable or tolerable for a given therapeutic indication.

Variability of the mixture from lot to lot
10 would be disadvantageous when developing a therapeutic pegylated protein product. In such development, predictability of biological activity is important. For example, it has been shown that in the case of nonselective conjugation of superoxide dismutase with
15 polyethylene glycol, several fractions of the modified enzyme were completely inactive (P. McGoff et al. Chem. Pharm. Bull. 36:3079-3091 (1988)). See also, Rose et al., Bioconjugate Chemistry 2: 154-159 (1991) which reports the selective attachment of the linker group
20 carbonylhydrazide to the C-terminal carboxyl group of a protein substrate (insulin). One cannot have such predictability if the therapeutic protein differs in composition from lot to lot. Some of the polyethylene glycol moieties may not be bound as stably in some
25 locations as others, and this may result in such moieties becoming dissociated from the protein. Of course, if such moieties are randomly attached and therefore become randomly dissociated, the pharmacokinetics of the therapeutic protein cannot be
30 precisely predictable.

Also highly desirable is a derivatized MGDF product wherein there is no linking moiety between the polymer moiety and the MGDF moiety. One problem with the above methods is that they typically require a
35 linking moiety between the protein and the polyethylene glycol molecule. These linking moieties may be

2167090

-14-

antigenic, which is also disadvantageous when developing a therapeutic protein.

A method involving no linking group is described in Francis et al., In: "Stability of protein pharmaceuticals: in vivo pathways of degradation and strategies for protein stabilization" (Eds. Ahern., T. and Manning, M.C.) Plenum, New York, 1991) Also, Delgado et al. "Coupling of PEG to Protein By Activation with Tresyl Chloride, Applications In Immunoaffinity Cell Preparation", In: Fisher et al., ed., Separations Using Aqueous Phase Systems, Applications In Cell Biology and Biotechnology, Plenum Press, N.Y., N.Y. 1989 pp. 211-213 involves the use of tresyl chloride, which results in no linkage group between the polyethylene glycol moiety and the protein moiety. This method may be difficult to use to produce therapeutic products as the use of tresyl chloride may produce toxic by-products.

Chamow et al., Bioconjugate Chem. 5: 133-140 (1994) report the modification of CD4 immunoadhesin with mono-methoxy-polyethylene glycol ("MePEG glycol") aldehyde via reductive alkylation. The authors report that 50% of the CD4-Ig was mePEG-modified by selective reaction at the α -amino group of the N-terminus. Id. at page 137. The authors also report that the *in vitro* binding capability of the modified CD4-Ig (to the protein gp 120) decreased at a rate correlated to the extent of MePEGylation. Ibid.

Thus, there is a need for MGDF derivatives, and, more particularly, a need for pegylated MGDF. There also exists a need for methods to carry out such derivatization.

-15-

Summary of the Invention

In one aspect, the present invention provides novel polypeptides that specifically promote megakaryocyte growth and/or development ("Mpl ligands" or "MGDFs") which are substantially free from (i.e., isolated from) other proteins (i.e., mammalian proteins in the case of an Mpl ligand obtained from a mammalian source). Such proteins may be purified from cell sources producing the factors naturally or upon induction with other factors. They may also be produced by recombinant genetic engineering techniques. Mpl ligands may further be synthesized by chemical techniques, or a combination of the above-listed techniques.

The Mpl ligands of this invention are obtainable in their native form from mammalian sources. Two exemplary Mpl ligands isolated from canine aplastic plasma are described in the examples section herein. However, it is demonstrated in other examples herein that closely related Mpl ligands are present in aplastic plasma from both human and porcine sources. Notably, the activity of each of the human, porcine, and canine Mpl ligands is specifically inhibitable by the soluble form of the murine Mpl receptor, demonstrating that all of these Mpl ligands (as well as those from other mammalian sources, including murine) are closely related both on structural and activity levels.

It is expected that human, porcine, and other mammalian Mpl ligands, may be isolated from natural sources by procedures substantially as detailed herein. See Example 10. Accordingly, this invention generally encompasses mammalian Mpl ligands, such as from dogs, pigs, humans, mice, horses, sheep, and rabbits. Particularly preferred Mpl ligands are those from dogs, pigs and humans.

2167090

-16-

In addition, genes encoding human Mpl ligands have been cloned from a human fetal kidney and liver libraries and sequenced, as set forth in the Example section below. Two human polypeptide sequences have
5 been determined to have activity in a cell-based assay (see Example 4). These sequences differ in their length, but have identity over a large stretch of their amino acid sequences. The identical portions have
10 referred to herein as Megakaryocyte Growth and Development Factors (MGDFs); all general references to Mpl ligands shall apply to those referred to herein as MGDFs and vice versa. By "MGDF polypeptide" is meant a polypeptide that has an activity to specifically
15 stimulate or inhibit the growth and/or development of megakaryocytes. Exemplary such polypeptides are disclosed herein.

The Mpl ligands of the present invention have been found to be specifically active in the
20 megakaryocyte lineage, augmenting maturation and/or proliferation of megakaryocytes, as demonstrated in the assays of Examples 2 and 4 below. By "specifically" is meant that the polypeptides exhibit biological activity to a relatively greater degree towards megakaryocytes as
25 compared to many other cell types. Those that are stimulatory towards megakaryocytes are expected to have an *in vivo* activity of stimulating the production of platelets, through the stimulation of maturation and differentiation of megakaryocytes.

30 Two preferred Mpl ligands from a canine source have apparent molecular weights of approximately 25 kd and 31 kd as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Both proteins are purified during
35 the same purification protocol which is detailed in the examples section below.

-17-

Two preferred human ligands, MGDF-1 and MGDF-2, are 332 and 174 amino acids in length, respectively, not including a 21 amino acid putative signal peptide. These sequences, and a third related molecule, MGDF-3
5 are shown in FIGs. 11 and 12.

Still a further aspect of the present invention are processes for isolating and purifying the Mpl ligands of the present invention or fragments thereof from mammalian sources, preferably whole blood,
10 serum or plasma. Aplastic blood, serum or plasma are especially preferred starting materials. Aplastic blood, serum or plasma may be obtained by a process involving irradiating a mammal with a radiation source such as cobalt-60 at a radiation level of about 400-800
15 rads so as to render them aplastic. Such a procedure is known in the art, as exemplified in the publications cited in Example 1 below. In the case of humans, irradiated blood, plasma, or serum may be obtained from a patient after radiation therapy, e.g., to treat
20 cancer.

Thereafter, the aplastic blood, serum or plasma is subjected to a purification process. The purification process provided by the present invention comprises the following key procedures: lectin affinity
25 chromatography and Mpl receptor affinity chromatography. Each of these procedures results in an approximately 300-500-fold purification of the 25 and 31 kd proteins from canine aplastic plasma. Other standard protein purification procedures may be included with the above
30 procedures to further purify the Mpl ligands of the present invention, such as those procedures detailed below.

Another aspect of the present invention includes polynucleotides that encode the expression of a
35 mammalian Mpl ligand protein. Such DNA sequences may include an isolated DNA sequence that encodes the

-18-

expression of mammalian Mpl ligand proteins as described herein. The DNA sequences may also include 5' and 3' mammalian non-coding sequences flanking the Mpl ligand coding sequence. The DNA sequences may further encode
5 an amino terminal signal peptide. Such sequences may be prepared by any known method, including complete or partial chemical synthesis. The codons may be optimized for expression in the host cell chosen for expression (e.g., *E. coli* or CHO cells).

10 Also provided by the present invention are recombinant DNA molecules, each comprising vector DNA and a DNA sequence encoding a mammalian Mpl ligand. The DNA molecules provide the Mpl ligand DNA in operative association with a regulatory sequence capable of
15 directing the replication and expression of Mpl ligand in a selected host cell. Host cells (e.g., bacterial, mammalian insect, yeast, or plant cells) transformed with such DNA molecules for use in expressing a recombinant Mpl ligand protein are also provided by the
20 present invention.

The DNA molecules and transformed cells of the invention are employed in another aspect, a novel process for producing recombinant mammalian Mpl ligand protein, or peptide fragments thereof. [In this process
25 a cell line transformed with a DNA sequence encoding expression of Mpl ligand protein or a fragment thereof (or a recombinant DNA molecule as described above) in operative association with a suitable regulatory or expression control sequence capable of controlling
30 expression of the protein is cultured under appropriate conditions permitting expression of the recombinant DNA. This claimed process may employ a number of known cells as host cells for expression of the protein. Presently preferred cell lines for producing Mpl ligand are
35 mammalian cell lines (e.g., CHO cells) and bacterial cells (e.g., *E. coli*).

-19-

For *E. coli* production of Mpl ligand, it is preferred to employ Met and Lys residues at the N-terminus of the protein to be expressed, since the yield of expression product is typically higher. A particularly preferred expression product is Met-Lys human MGDF having a total of 165 amino acids (i.e., Met-Lys attached to the N-terminus of 1-163 MGDF (MGDF-11; amino acids 1-163 of SEQ ID NO:25) After purification of the product expressed in a bacterial cell such as *E. coli*, the terminal Met-Lys residues may be removed by treatment with an enzyme such as a dipeptidase (e.g., cathepsin C).

The expressed Mpl ligand protein is then harvested from the host cell, cell lysate or culture medium by suitable conventional means. The conditioned medium may be processed through the same purification steps or modifications thereof as used to isolate the Mpl ligand from aplastic plasma. (See Example 7).

In a still further aspect of the present invention, there are provided recombinant Mpl ligand proteins. These proteins are substantially free from other mammalian materials, especially proteins. The Mpl ligand proteins of this invention are also characterized by containing one or more of the physical, biochemical, pharmacological or biological activities described herein.

The present invention also relates to chemically modified MGDF comprised of a MGDF protein moiety connected to at least one water soluble polymer, and methods for the preparation and use of such compositions. In particular, the present invention includes chemically modified MGDF wherein the MGDF species is reacted with reactive polyethylene glycol molecules so as to attach PEG to MGDF. Such attachment may be accomplished by pegylation reactions discussed herein, such as acylation or alkylation. Acylation or

-20-

alkylation with PEG may be carried out under conditions whereby the major product is monopegylated or polypegylated. Polypegylation generally involves attachment of PEG to the ϵ -amino groups of lysine residues and may additionally involve pegylation at the N-terminus of the polypeptide. Monopegylation preferably involves attachment of PEG to the α -amino group at the N-terminus of the protein. The yield and homogeneity of such monopegylation reaction may be enhanced via a type of reductive alkylation which selectively modifies the α -amino group of the N-terminal residue of an MGDF protein moiety, thereby providing for selective attachment of a water soluble polymer moiety at the N-terminus of the protein. This provides for a substantially homogeneous preparation of polymer/MGDF protein conjugate molecules as well as (if polyethylene glycol is used) a preparation of pegylated MGDF protein molecules having the polyethylene glycol moiety directly coupled to the protein moiety.

Another aspect of this invention provides pharmaceutical compositions containing a therapeutically effective amount of isolated naturally-occurring or recombinant Mpl ligand, which may be derivatized with a water soluble polymer such as polyethylene glycol, along with a pharmaceutically acceptable carrier, diluent, or excipient. These pharmaceutical compositions may be employed in methods for treating disease states or disorders characterized by a deficiency of megakaryocytes and/or platelets as well as an *in vivo* deficiency of the Mpl ligand. They may also be employed prophylactically to ameliorate expected megakaryocyte or platelet deficiencies (e.g., due to surgery).

Thus, the Mpl ligands of the present invention may be employed in the treatment of aplastic anemias, e.g., to augment production of platelets in patients having impaired platelet production (such as AIDS

-21-

patients or patients undergoing cancer chemotherapy).
Mpl ligand may be used to treat blood disorders such as
thrombocytopenia. Mpl ligand may be used as an
adjunctive therapy for bone marrow transplant patients.

5 Such patients could be human or another mammal. Mpl
ligand from one species is also expected to be useful in
another species.

A further aspect of the invention, therefore,
is a method for treating these and other pathological
10 states resulting from a deficiency of platelets by
administering to a patient a therapeutically effective
amount of a pharmaceutical composition as described
above. These therapeutic methods may include
administration, simultaneously or sequentially with Mpl
15 ligand, an effective amount of at least one other
megakaryocyte colony stimulating factor, a cytokine
(e.g., EPO), a soluble Mpl receptor, hematopoietin,
interleukin, growth factor, or antibody.

Still another aspect of the present invention
20 provides antibodies (e.g., polyclonal, monoclonal,
humanized, and recombinant), and antibody fragments,
directed against (i.e., reactive with) a mammalian Mpl
ligand or a ligand fragment. As part of this aspect,
therefore, the invention includes cells capable of
25 secreting such antibodies (e.g., hybridomas in the case
of monoclonal antibodies) and methods for their
production and use in diagnostic or therapeutic
procedures.

Another aspect of the invention is an assay of
30 a body fluid for the presence of Mpl ligand. Such an
assay could employ antibodies that specifically
recognize an Mpl ligand, in a single antibody or
"sandwich" format. Such an assay could be used to
determine whether a patient needs external
35 administration of Mpl ligand and/or whether such patient
is likely to experience a platelet deficiency or

-22-

disorder. Such assays could be included in a kit format, including positive and negative controls, antibody(ies), and other standard kit components.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of preferred embodiments thereof.

Brief Description of the Figures

10

Numerous features and advantages of the present invention will become apparent upon review of the figures, wherein:

15

FIG. 1 depicts an overview of development and maturation of megakaryocytes and platelets.

20

FIG. 2 demonstrates that soluble murine Mpl receptor substantially completely inhibits the ability of plasma from irradiated dogs ("aplastic canine" or "APK9"), to induce megakaryocyte development. The assay for megakaryocyte development was that described in Example 2.

25

FIG. 3 shows that an activity enriched from APK9 by lectin affinity and Mpl receptor affinity chromatography procedures ("Mpl ligand") stimulates the growth of 1A6.1 cells and that soluble murine Mpl receptor blocks that growth.

30

FIG. 4 shows an overview of the purification steps involved in purifying the 25 and 31 kd forms of the canine Mpl receptor from aplastic canine plasma.

35

FIG. 5 shows the purification of Mpl ligand by reversed phase HPLC (RP-HPLC). Fraction 21 contains

-23-

highly purified 31 kd Mpl ligand; fraction 22 contains a mixture of the 31 kd and 25 kd Mpl ligands; and fraction 23 contains highly purified 25 kd Mpl ligand.

5 FIG. 6 shows a comparison of Mpl ligand activities in reverse phase HPLC (C4 column) fractions that contain the 25 and/or 31 kd Mpl ligand proteins.

10 FIG. 7 shows the number of megakaryocytes produced from cultures of CD34-selected peripheral blood cells stimulated with APK9, Mpl ligand and various other factors.

15 FIG. 8 shows the number of total leukocytes produced from cultures of CD34-selected peripheral blood cells stimulated with APK9, Mpl ligand and various other factors.

20 FIG. 9 shows the percentages of megakaryocytes that are produced in cultures of CD34-selected peripheral blood cells stimulated with APK9, Mpl ligand and various other factors.

25 FIG. 10 shows that human IL-3 is not involved in Mpl ligand-induced megakaryocyte development.

 FIG. 11 shows the cDNA and deduced amino acid sequences of human MGDF-1 and MGDF-2.

30 FIG. 12 shows the cDNA and deduced amino acid sequences of human MGDF-3.

35 FIG. 13 shows a comparison between MGDF-1 and MGDFs (Mpl ligands) from a canine source (A) and a murine source (B).

2167090

-24-

FIG. 14 shows an example of MGDF acylation using N-hydroxysuccinimidyl (NHS) active esters of mono-methoxy-polyethylene glycol to result in a poly-pegylated product.

5

FIG. 15 shows an example of nonspecific MGDF reductive alkylation using mono-methoxy-polyethylene glycol aldehydes to result in a poly-pegylated product.

10

FIG. 16 shows an example of site-specific MGDF reductive alkylation at the α -amino group of the N-terminal residue using mono-methoxy-polyethylene glycol aldehydes to result in a substantially mono-pegylated product.

15

FIG. 17 shows size exclusion (SEC) HPLC analysis of MePEG-MGDF conjugates prepared using activated derivatives of MW 20kDa MePEG:

A. poly-MePEG-MGDF conjugate prepared by MGDF acylation with NHS ester of MePEG (PEG 11)

B. poly-MePEG-MGDF conjugate prepared by MGDF alkylation with MePEG aldehyde (PEG 20);

C. mono-MePEG-MGDF conjugate prepared by MGDF alkylation with MePEG aldehyde (PEG 16).

25

FIG. 18 shows platelet counts from mice treated with recombinant human MGDF: open diamond = CHO-derived 22-353 MGDF; open circles = unpegylated *E. coli* 22-184 MGDF (i.e., 1-163 MGDF); and closed circles = pegylated *E. coli* 22-184 MGDF.

30

FIG. 19 shows a purification flow chart for r-HuMGDF.

35

FIG. 20 shows the effect of r-HuMGDF (*E. coli* 1-163) on platelet counts in a murine carboplatin model.

-25-

Balb/c mice were intraperitoneally injected with a single dose of carboplatin (1.25 mg/mouse) at Day 0. The excipient alone group did not receive carboplatin. After twenty-four hours, carboplatin-treated animals were subcutaneously injected with either excipient or with 100 ug/kg r-HuMGDF daily for the remainder of the study. (n=10 for each group; 5 animals were bled at every other time point).

FIG. 21 shows the effect of r-HuMGDF (*E. coli* 1-163) on platelet counts in mice treated with irradiation. Balb/c mice were irradiated with a single dose of 500 rads gamma-irradiation (Cesium source) at Day 0. The excipient alone group was not irradiated. After twenty-four hours, irradiated animals were subcutaneously injected with either excipient or with 100 ug/kg r-HuMGDF daily for the remainder of the study. (n=8 for each group; 4 animals were bled at every other time point).

FIG. 22 shows the effect of r-HuMGDF (*E. coli* 1-163) on platelet counts in mice treated with a combination of irradiation and carboplatin. Balb/c mice were irradiated with a single dose of 500 rads gamma-irradiation (Cesium source) and given carboplatin (1.25 mg/mouse) at Day 0. After twenty-four hours, the compromised animals were subcutaneously injected with either excipient or with 100 ug/kg r-HuMGDF daily for the remainder of the study (n=8 each group). Without r-HuMGDF support, most of the animals do not survive this study. In the control group, 1 of 8 animals survived. In the treated group, 8 of 8 animals survived.

FIG. 23 shows the effect of r-HuMGDF (*E. coli* 1-163) on irradiation-induced thrombocytopenia in rhesus monkeys. Rhesus monkeys were subjected to irradiation

2167090

-26-

(700 cGy Co-80). r-HuMGDF (n=3) or human serum albumin (n=9) (each at 25 ug/kg/day) were administered subcutaneously for 18 consecutive days starting 24 hours after irradiation. Blood cell analyses were performed with an electronic blood cell analyzer. Each symbol represents the average value (+/- sem).

FIG. 24 shows the effects of pegylated and glycosylated r-HuMGDF on platelet counts in mice treated with carboplatin and irradiation. Mice were subjected to the combination of carboplatin and irradiation as described for the studies performed for FIG. 22. Subcutaneous injections of the indicated preparation of r-HuMGDF (50 ug/kg/day) were given daily for the length of the study starting 24 hours after the insult. Blood cell counts were taken on the indicated day using an electronic cell counter (Sysmex, Baxter).

FIG. 25 shows the synthetic gene sequence for recombinant human MGDF, amino acids 1-163, having *E. coli* optimized codons.

Detailed Description of the Invention

Additional aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following description, which details the practice of the invention.

The novel mammalian megakaryocyte growth promoting, and/or platelet producing factors, referred to as Mpl ligands, provided by the present invention are homogeneous proteins substantially free of association with other proteinaceous materials. Preferably, the proteins are about 90% free of other proteins, particularly preferably, about 95% free of other proteins, and most preferably about $\geq 98\%$ free of other

-27-

proteins. These proteins can be produced via recombinant techniques to enable large quantity production of pure, active Mpl ligand useful for therapeutic applications. Alternatively such proteins
5 may be obtained in a homogeneous form from mammalian aplastic blood, plasma or serum, or from a mammalian cell line secreting or expressing an Mpl ligand. Further, Mpl ligand or active fragments thereof may be chemically synthesized.

10 In general, by "Mpl ligands" as used in connection with the present invention is meant the Mpl ligands disclosed herein as well as active fragments and variants thereof, which are described in greater detail below.

15 Two preferred Mpl ligands from a canine source have apparent molecular weights of approximately 25 kd and 31 kd as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Both proteins are purified during
20 the same purification protocol which is detailed in the examples section below. Thus, for example, both of these Mpl ligands bind wheat germ lectin and immobilized Mpl receptor. The 25 kd form includes an amino acid sequence as follows:

25 Ala-Pro-Pro-Ala-Xaa-Asp-Pro-Arg-Leu-Leu-Asn-Lys-Met-Leu-Arg-Asp-Ser-His-Val-Leu-His-Xaa-Arg-Leu-Xaa-Gln-Xaa-Pro-Asp-Ile-Tyr (SEQ ID NO: 1).

30 The 31 kd form includes an amino acid sequence as follows:

Ala-Pro-Pro-Ala-Xaa-Asp-Pro-Arg-Leu-Leu-Asn-Lys-Met-Leu-Arg-Asp-Ser-His-Val-Leu-His (SEQ ID NO: 2).

35 The "Xaa" amino acids shown in SEQ ID NOS: 1 and 2 are

2167090

-28-

not known with certainty, but are expected to be cysteine, serine, threonine, or (less likely) tryptophan.

It can be seen from the above sequences that the 31 kd ligand comprises at least a portion of the 25 kd form. In particular, the first 21 amino acids of the 31 kd protein are exactly the same as those of the 25 kd protein. This evidence, and especially the fact that both proteins have activity in the Mpl ligand activity assays presented herein, leads to the conclusion that both proteins are very closely related in terms of structure and activity. It is likely that the 31 kd form of the protein differs from the 25 kd form in differential C-terminal sequence, differential glycosylation and/or differential splicing of the gene encoding the proteins.

In addition to the above sequence information, another sequence was determined during sequencing of the 25 kd band prior to the final purification step (using reverse phase HPLC). This sequence was found associated with the 25 kd band under non-reducing conditions but not reducing conditions, implying that it is the result of cleavage into two portions (e.g., by a protease) of the 25 kd protein, which portions are held together by a disulfide bond. This sequence is:

Thr-Gln-Lys-Glu-Gln-Thr-Lys-Ala-Gln-Asp-Val-Leu-Gly-Ala-Val-Ala-Leu (SEQ ID NO: 3)

Although the precise location of SEQ ID NO: 3 in the sequence of the 25 kd protein is unclear, analogy with other cytokines, such as erythropoietin, supports the possibility that the sequence occurs around amino acid number 114 in the 25 kd protein. It should be noted that it is likely, although unproven, that SEQ ID NO: 3 also occurs in the 31 kd protein, probably again

-29-

starting around amino acid number 114. This sequence information is discussed in additional detail in Example 7.

Since the initial purification experiments of the canine ligands, summarized above, a gene encoding a canine ligand has now been cloned. As a result, the full length amino acid sequence of this canine ligand has been determined to be that set forth in FIG. 13A. Based on molecular weight calculations, it is predicted that the 25 kd and 31 kd canine ligands are C-terminal processed forms of the full-length ligand shown in FIG. 13A. Additionally, a murine Mpl ligand has been obtained having the sequence set forth in FIG. 13B.

Such purified ligands may also be characterized by specific activity in the human megakaryocyte assay of Example 2 of at least about 5.7×10^9 megakaryocyte units/mg. A megakaryocyte unit is defined as that amount of material that results in the production of as many megakaryocytes as 1 microliter of APK9 standard control using the assay described in Example 2.

Such purified ligands are also characterized by a specific activity in the Mpl-dependent cell growth assay of Example 4 of at least about 6.9×10^9 cell growth units/mg. A "cell growth unit" is defined as the amount of ligand required to result in the growth of 200 1A6.1 cells in the assay of Example 4.

The following Table 1 shows additional specific calculations of activity for actual purified canine Mpl ligands prepared in accordance with this invention:

-30-

Table 1

	Mpl <u>Ligand</u>	1A6.1 assay <u>(units/mg)</u>	Human Meg assay <u>(units/mg)</u>
5	31 kd	6.52×10^9	5.7×10^9
	25 kd	10.5×10^9	14×10^9

Summarizing the above information, some exemplary Mpl ligands of the present invention are characterized by one or more of the following biochemical and biological properties:

(a) such Mpl ligands are isolated from canine aplastic plasma;

(b) such Mpl ligands have apparent molecular weights of approximately 25 kd or 31 kd as determined by 12-14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions;

(c) the Mpl ligands comprise the following amino acid sequences:

SEQ ID NO: 1, in the case of the 25 kd protein; or

SEQ ID NO: 2, in the case of the 31 kd protein;

(d) the Mpl ligands additionally comprise the amino acid sequence SEQ ID NO: 3 (particularly preferably in the 25 kd protein);

(e) the Mpl ligands bind to wheat germ lectin;

(f) the Mpl ligands bind to immobilized soluble murine Mpl receptor;

(g) the Mpl ligand activity can be inhibited *in vitro* by soluble Mpl receptor; and

(h) the Mpl ligands bind to an anion exchange column at a pH of about 8-9.

The biological activities of preferred Mpl

-31-

ligands of the present invention are demonstrated by their ability to specifically stimulate the growth and development of megakaryocytes in the megakaryocyte growth promoting assay of Example 2. In this assay, MPL
5 ligand stimulates the differentiation of human peripheral blood CD34⁺ (i.e., CD-34 cells selected by immunoadsorption) cells during an 8 day culture period. Megakaryocytes are identified by staining with specific anti-platelet antigen antibodies and counted
10 microscopically. MPL ligand also stimulates the growth of the factor-dependent cell line, 1A6.1. In the absence of MPL ligand, the cells will die. 1A6.1 cell number is assessed after 2 days in culture with MPL ligand.

15 The Mpl ligands described above have specific activities as described in Table 1 above.

Sources of the Mpl ligands have been determined to be aplastic mammalian blood, plasma, or serum. However, the source of such ligands is not
20 expected to be limited to such known sources and may include other mammalian body fluids, cells obtained therefrom, etc.

The purification of native Mpl ligands from mammalian sources is based on two key purification
25 steps:

(a) lectin affinity chromatography, preferably using wheat germ agglutinin; and

(b) immobilized Mpl receptor affinity chromatography.

30 Additional steps may be included to further purify the protein, such as ion exchange chromatography, gel filtration chromatography, and reverse phase chromatography.

The purification techniques actually employed
35 in obtaining Mpl ligand from canine aplastic plasma comprise the following steps (See, Example 7):

-32-

(a) lectin affinity chromatography (wheat germ agglutinin chromatography is especially preferred);

(b) soluble Mpl receptor (Mpl-X) affinity chromatography (preferably, using immobilized murine Mpl-X);

(c) ion (anion or cation) exchange chromatography (preferably, anion exchange chromatography; particularly preferably using a Mono Q* column);

(d) gel filtration chromatography under dissociative conditions (preferably, using Superdex 200* plus SDS); and

(e) reverse phase chromatography (preferably, using a C-4* column).

Homogeneous mammalian Mpl ligand, including the human ligand, may be obtained by applying the above purification procedures to aplastic blood, serum, or plasma or other sources of mammalian Mpl ligand, e.g., cell or tissue sources. The steps are not required to be in a particular sequence, but the listed sequence is preferred. Procedures for culturing a cell (or tissue) source which may be found to produce Mpl ligand are known to those of skill in the art and may be used, for example, to expand the supply of starting material.

Mpl ligand or one or more peptide fragments thereof may also be produced via recombinant techniques. To obtain the DNA sequence for a particular Mpl ligand, the purified Mpl ligand material is reduced and optionally digested with a protease such as trypsin. Enzymatic fragments are isolated and sequenced by conventional techniques. Alternatively, as exemplified in the examples herein, the intact purified protein may be sequenced directly to the extent possible based on the quantity of protein available and then the sequenced region may be used analogously to the sequenced tryptic fragments in the following procedure. Oligonucleotide

*Trademark

-33-

probes are synthesized using the genetic code to predict all possible sequences that encode the amino acid sequences of the sequenced fragment(s). Preferably, several degenerate sequences are generated as probes.

5 The Mpl ligand gene is identified by using these probes to screen a mammalian genomic library or other source. Alternatively, the mRNA from a cell source of Mpl ligand can be used to make a cDNA library which can be screened with the probes to identify the cDNA encoding the Mpl

10 ligand polypeptide. Further, the PCR technique may be used to extend the cDNA sequence, using appropriate primers.

Using these probes to screen a genomic library, a DNA clone is obtained. To obtain a full

15 length clone, probes based on the obtained DNA sequence may be employed to rescreen the library and hybridize to the full length Mpl ligand DNA sequence.

The human cDNA for Mpl ligand can also be obtained by subcloning a full length human genomic clone

20 into an expression vector, transfecting it into COS cells, preparing a cDNA library from these transfected COS cells and screening by hybridization for Mpl ligand cDNA. Once the entire cDNA is identified, it or any portion of it that encodes an active fragment of Mpl

25 ligand, can be introduced into any one of a variety of expression vectors to make an expression system for Mpl ligand or one or more fragments thereof.

By such use of recombinant techniques, preferred DNA sequences encoding the Mpl ligand

30 polypeptide are obtained. The present invention also encompasses these DNA sequences, free of association with DNA sequences encoding other proteins (i.e., isolated), and coding for expression of Mpl ligand polypeptides with an Mpl ligand activity (e.g.,

35 megakaryocyte growth and/or development). These DNA sequences include those sequences encoding all or a

-34-

fragment of Mpl ligand and those sequences which hybridize, preferably under stringent hybridization conditions to the cDNA sequences [See, T. Maniatis et. al., *Molecular Cloning* (A Laboratory Manual); Cold Spring Harbor Laboratory (1982), pages 387 to 389].

Exemplary stringent hybridization conditions are hybridization in 4 X SSC at 62-67° C., followed by washing in 0.1 X SSC at 62-67° C. for approximately an hour. Alternatively, exemplary stringent hybridization conditions are hybridization in 45-55% formamide, 4 X SSC at 40-45°C.

DNA sequences which hybridize to the sequences for Mpl ligand under relaxed hybridization conditions and which encode Mpl ligand peptides having Mpl ligand biological properties also encode novel Mpl ligand polypeptides of this invention. Examples of such relaxed stringency hybridization conditions are 4 X SSC at 45-55°C. or hybridization with 30-40% formamide at 40-45° C. For example, a DNA sequence which shares regions of significant homology, e.g., sites of glycosylation or disulfide linkages, with the sequences of Mpl ligand and encodes a protein having one or more Mpl ligand biological properties clearly encodes an Mpl ligand polypeptide even if such a DNA sequence would not stringently hybridize to the Mpl ligand sequence(s).

Allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) of DNA sequences encoding the peptide sequences of Mpl ligand are also included in the present invention, as well as analogs or derivatives thereof. Similarly, DNA sequences which code for Mpl ligand polypeptides but which differ in codon usage due to the degeneracies of the genetic code or variations in the DNA sequence of Mpl ligand which are caused by point mutations or by induced modifications to enhance the activity, half-life or

-35-

production of the polypeptides encoded thereby are also encompassed in the invention.

A cloning procedure as set forth in Example 11 below was followed and resulted in the amino acid and cDNA sequences of the human proteins MGDF-1, MGDF-2, and MGDF-3 disclosed herein. MGDF-1 is shown as amino acids 22-353 in FIG. 11 and contains 332 amino acids. MGDF-2 is a truncated portion of MGDF-1, and is shown as amino acids 22-195 in FIG. 11. MGDF-2 therefore contains 174 amino acids. MGDF-3 is shown as amino acids 22-286 in FIG. 12 and contains 265 amino acids. In each MGDF disclosed herein, the molecule including the signal peptide, shown as amino acids 1-21 in both FIGS. 11 and 12, is also part of the present inventive polypeptides, but it is preferably removed for megakaryocyte growth and development activity to be exhibited. In summary, MGDFs 1-3 are defined as follows:

	MGDF-1	amino acids	22-353	FIG. 11
20	MGDF-2	amino acids	22-195	FIG. 11
	MGDF-3	amino acids	22-286	FIG. 12.

In the assays presented herein, MGDF-1 and MGDF-2 were active whereas MGDF-3 was not.

Based on the activity data presented herein, it is hypothesized that human MGDF is expressed *in vivo* as a substantially inactive or less active precursor polypeptide that contains variable C-terminal amino acids. Upon cleavage of the C-terminal amino acids (as well as the signal peptide), the processed form(s) of the molecule retain activity or become more active. In view of the above hypothesis, it is believed that MGDF-1 may require processing (e.g., cleavage with a protease) in order to exhibit its activity. The fact that a truncated form of MGDF-1 (i.e., MGDF-2) is active supports this hypothesis.

-36-

Conditioned medium from human kidney 293 cells (Invitrogen) transfected with the MGDF-1 gene does demonstrate activity in the cell assay of Example 4 below. On the other hand, in other cell lines, e.g., 32 D cells, no activity was seen for this molecule. It is hypothesized that this may mean that 293 cells are able to process the MGDF-1 molecule, presumably by truncation, so that the molecule primarily responsible for the activity is a truncated form, whereas the 32 D cells are unable to process the molecule.

In view of the above hypothesis, various active molecules may result from truncations of the sequence set forth as MGDF-1 (FIG. 11). Structural features conserved among the cytokine family, such as erythropoietin (EPO), include four α -helical bundles and four cysteines. Referring to the MGDF-1 sequence, Cys 172 is the most C-terminal element of these evolutionarily conserved and functionally essential structures. Therefore, preferred truncation variants of MGDF-1 are any of those that have C-terminal truncations from amino acid 173 to 353 (along with cleavage of the signal peptide). Preferably, the sequence of MGDF-1 will have removed from it from 50 to 185 amino acids from the C-terminus, particularly preferably, from 90 to 172 amino acids from the C terminal. As disclosed herein, the signal peptide is thought to be 21 amino acids in length; however, the signal peptide may have 23 amino acids, based on the sequence of MGDF-1. Accordingly, polypeptides corresponding to those presented herein but which start at position 24 of FIG. 11 or 12 are also specifically contemplated.

The following are some specific preferred variants of MGDF-1 that may exhibit activity (i.e., the ability to promote the growth of megakaryocytes and/or platelets; or inhibitory/ stimulatory activity towards the natural receptor):

-37-

5	MGDF-4	amino acids	22-172	FIG. 11
	MGDF-5	amino acids	22-177	FIG. 11
	MGDF-6	amino acids	22-191	FIG. 11
	MGDF-7	amino acids	22-198	FIG. 11
	MGDF-8	amino acids	22-265	FIG. 11
	MGDF-11	amino acids	22-184	FIG. 11

10 In some clones, amino acids 133-136 in the MGDF-1 sequence were absent, so that sequences corresponding to those set forth above, but in which these amino acids are missing (and the C-terminus amino acid number adjusted down by 4) may also be active.

15 In one clone, which had a termination codon at position 192, an Ala residue was found instead of a Thr residue as shown at position 191 in FIG. 11. Therefore, the invention includes variants of MGDF molecules in which position 191 is Ala instead of Thr.

20 MGDF-3 results from removal of a sequence referred to herein as IVS-5 (Intervening Sequence-5) since this sequence is spliced within the fifth exon in the sequence. Since the 5' end of IVS-5 occurs within a codon, its removal results in a frame-shift in the remaining sequence of MGDF, which can be seen to occur
25 starting at position 160 of MGDF-3 to the end of the molecule.

No activity has yet been found for MGDF-3 itself upon transfection into 293 cells and testing the resulting conditioned medium for activity in the cell-
30 based assay of Example 4. Apparently, unlike MGDF-1, 293 cells are unable to process MGDF-3 to an active form. Nevertheless, based on the truncation hypothesis set forth above in connection with MGDF-1, truncation of C-terminal amino acids from MGDF-3 may also result in
35 activity. For example, C-terminal truncation of MGDF-3 of from 40 to 102 amino acids may result in activity.

2167090

-38-

Preferably, from 50 to 90 amino acids are removed. Two specific preferred variants of MGDF-3 are:

	MGDF-9	22-179	FIG. 12
5	MGDF-10	22-190	FIG. 12

In all of the Mpl ligands disclosed herein, including the exemplary MGDFs set forth above, a methionyl residue may be present at the N-terminus, especially when such polypeptides are expressed in bacterial host cells.

Mpl ligand polypeptides may also be produced by known conventional chemical synthesis. Methods for constructing the polypeptides of the present invention by synthetic means are known to those of skill in the art. The synthetically-constructed Mpl ligand polypeptide sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with Mpl ligand polypeptides may possess Mpl ligand biological properties in common therewith. Thus, they may be employed as biologically active or immunological substitutes for natural, purified Mpl ligand polypeptides in therapeutic and immunological processes.

Modifications in the peptides or DNA sequences encoding Mpl ligand can be made by one skilled in the art using known techniques. Modifications of interest in the Mpl ligand sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences. Mutagenesis techniques for such replacement, insertion or deletion are well known to one skilled in the art. [See, e.g., U.S. Pat. No. 4,518,584.] Conservative changes in from 1 to 20 amino acids are preferred. Preferred peptides may be generated by proteolytic enzymes, or by direct chemical synthesis. Such variants are included within the

-39-

meaning of Mpl ligand polypeptides and polynucleotides of the present invention.

Specific mutations of the sequences of the Mpl ligand polypeptide may involve modifications of a glycosylation site (e.g., serine, threonine, or asparagine). The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either Asn-Xaa-Thr or Asn-Xaa-Ser, where Xaa can be any amino acid other than Pro. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Expression of such altered nucleotide sequences produces variants which are not glycosylated at that site.

25

Additional Analogs/Derivatives of MGDF

Other analogs and derivatives of the sequences of MGDF (Mpl ligands), which may retain MGDF (Mpl ligand) activity in whole or in part may also be prepared by one of skill in the art given the disclosures herein. Such modifications are also encompassed by this invention.

More particularly, the present invention also broadly includes chemically modified MGDF compositions and methods of making and using them. The present

2167090

-40-

disclosure reveals that it is possible to modify MGDF and to enhance its properties.

In one aspect, the present invention relates to an MGDF product comprising an MGDF protein linked to
5 at least one water soluble polymer moiety.

In another aspect, the present invention relates to an MGDF product wherein said MGDF protein is linked to at least one polyethylene glycol molecule.

In another aspect, the present invention
10 relates to MGDF molecules attached to at least one polyethylene glycol molecule via an acyl or alkyl linkage.

Pegylation of MGDF may be carried out by any of the pegylation reactions known in the art. See, for
15 example: *Focus on Growth Factors 3* (2): 4-10 (1992); EP 0 154 316; EP 0 401 384; and the other publications cited herein that relate to pegylation. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene
20 glycol molecule (or an analogous reactive water-soluble polymer). These preferred means for derivatization with polyethylene glycol will now be discussed in greater detail.

25 Acylation

Pegylation by acylation generally involves reacting an active ester derivative of polyethylene glycol (PEG) with an MGDF protein. Any known or subsequently discovered reactive PEG molecule may be
30 used to carry out the pegylation of MGDF. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide ("NHS"). As used herein, "acylation" is contemplated to include without limitation the following types of linkages between MGDF and a water
35 soluble polymer such as PEG: amide, carbamate, urethane, and the like. See *Bioconjugate Chem.* 5: 133-140 (1994).

-41-

Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions such as temperature, solvent, and pH that would inactivate the MGDF species to be modified. Reaction conditions that apply generally to pegylation of MGDFs will be described below. An exemplary reaction with an NHS ester of monomethoxy-PEG is depicted in FIG. 14.

Pegylation by acylation will generally result in a poly-pegylated MGDF product, wherein the lysine ϵ -amino groups are pegylated via an acyl linking group. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., $\geq 95\%$) mono, di- or tri-pegylated. However, some species with higher degrees of pegylation (up to the maximum number of lysine ϵ -amino acid groups of MGDF plus one α -amino group at the amino terminus of MGDF) will normally be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture, particularly unreacted species, by standard purification techniques, including, among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

Alkylation

Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with a protein such as MGDF in the presence of a reducing agent. As with acylation, discussed above, the reaction conditions are described below.

Pegylation by alkylation can also result in poly-pegylated MGDF. An exemplary reductive alkylation reaction with MGDF to yield a polypegylated product is shown in FIG. 15. In addition, one can manipulate the

-42-

reaction conditions as described herein to favor
pegylation substantially only at the α -amino group of
the N-terminus of the MGDF species (i.e., a mono-
pegylated species). An exemplary reductive alkylation
5 reaction with MGDF to yield a monopegylated product is
shown in FIG. 16. In either case of monopegylation or
polypegylation, the PEG groups are preferably attached
to the protein via a $-\text{CH}_2\text{-NH-}$ group. With particular
reference to the $-\text{CH}_2\text{-}$ group, this type of linkage is
10 referred to herein as an "alkyl" linkage.

Derivatization via reductive alkylation to
produce a monopegylated product exploits differential
reactivity of different types of primary amino groups
(lysine versus the N-terminal) available for
15 derivatization in MGDF. The reaction is performed at a
pH (see below) which allows one to take advantage of the
pK_a differences between the ϵ -amino groups of the lysine
residues and that of the α -amino group of the N-terminal
residue of the protein. By such selective
20 derivatization, attachment of a water soluble polymer
that contains a reactive group such as an aldehyde, to a
protein is controlled: the conjugation with the polymer
takes place predominantly at the N-terminus of the
protein and no significant modification of other
25 reactive groups, such as the lysine side chain amino
groups, occurs. In one important aspect, the present
invention provides for a substantially homogeneous
preparation of monopolymer/MGDF protein conjugate
molecules (meaning MGDF protein to which a polymer
30 molecule has been attached substantially only (i.e., $\geq$
95%) in a single location). More specifically, if
polyethylene glycol is used, the present invention also
provides for pegylated MGDF protein lacking possibly
antigenic linking groups, and having the polyethylene
35 glycol molecule directly coupled to the MGDF protein.

Thus, in a preferred aspect, the present

-43-

invention relates to pegylated MGDF, wherein the PEG group(s) is (are) attached via acyl or alkyl groups. As discussed above, such products may be mono-pegylated or poly-pegylated (e.g., containing 2-6, preferably 2-5, 5 PEG groups). The PEG groups are generally attached to the protein at the α or ϵ amino groups of amino acids, but it is also contemplated that the PEG groups could be attached to any amino group attached to the protein, which is sufficiently reactive to become attached to a 10 PEG group under suitable reaction conditions.

The polymer molecules used in both the acylation and alkylation approaches may be selected from among water soluble polymers or a mixture thereof. The polymer selected should be water soluble so that the 15 protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer selected should be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, preferably, 20 so that the degree of polymerization may be controlled as provided for in the present methods. A preferred reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see, U.S. Patent 25 5,252,714). The polymer may be branched or unbranched. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. The water soluble polymer may be selected from the group consisting of, for example, polyethylene 30 glycol, monomethoxy-polyethylene glycol, dextran, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol. For the acylation 35 reactions, the polymer(s) selected should have a single reactive ester group. For the present reductive

2167090

-44-

alkylation, the polymer(s) selected should have a single reactive aldehyde group. Generally, the water soluble polymer will not be selected from naturally-occurring glycosyl residues since these are usually made more
 5 conveniently by mammalian recombinant expression systems. The polymer may be of any molecular weight, and may be branched or unbranched.

A particularly preferred water-soluble polymer for use herein is polyethylene glycol, abbreviated PEG.
 10 As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol.

As employed herein, MGDF is defined as
 15 including any of the various forms of MGDF described herein. For example, full-length or truncated, glycosylated or nonglycosylated forms of MGDF are all included. The following are preferred MGDF molecules to be derivatized (in each case the numbering refers to the
 20 amino acids numbered in accordance with FIG. 11):

	MGDF-1	amino acids	22-353	FIG. 11
	MGDF-2	amino acids	22-195	FIG. 11
	MGDF-4	amino acids	22-172	FIG. 11
25	MGDF-11	amino acids	22-184	FIG. 11
	MGDF-12	amino acids	27-353	FIG. 11
	MGDF-13	amino acids	27-195	FIG. 11
	MGDF-14	amino acids	27-172	FIG. 11
	MGDF-15	amino acids	27-184	FIG. 11

30

The above-preferred species may be glycosylated, non-glycosylated, or de-glycosylated, preferably non-glycosylated. They may be made recombinantly in either bacterial (e.g., *E. coli*) or mammalian (e.g., CHO)
 35 cells.

The following are particularly preferred sub-

-45-

groups of chemically derivatized molecules of this invention (in each case, they are mono- or poly-, e.g., 2-4, PEG moieties, attached via an acyl or alkyl group):

- 5 pegylated MGDF-11
- pegylated MGDF-4
- pegylated MGDF-2.

 In general, chemical derivatization may be
10 performed under any suitable condition used to react a
biologically active substance with an activated polymer
molecule. Methods for preparing pegylated MGDF will
generally comprise the steps of (a) reacting an MGDF
15 polypeptide with polyethylene glycol (such as a reactive
ester or aldehyde derivative of PEG) under conditions
whereby MGDF becomes attached to one or more PEG groups,
and (b) obtaining the reaction product(s). In general,
the optimal reaction conditions for the acylation
reactions will be determined case-by-case based on known
20 parameters and the desired result. For example, the
larger the ratio of PEG: protein, the greater the
percentage of poly-pegylated product.

 Reductive alkylation to produce a
substantially homogeneous population of mono-
25 polymer/MGDF protein conjugate molecule will generally
comprise the steps of: (a) reacting an MGDF protein with
a reactive PEG molecule under reductive alkylation
conditions, at a pH suitable to permit selective
modification of the α -amino group at the amino terminus
30 of said MGDF protein; and (b) obtaining the reaction
product(s).

 For a substantially homogeneous population of
mono-polymer/MGDF protein conjugate molecules, the
reductive alkylation reaction conditions are those which
35 permit the selective attachment of the water soluble
polymer moiety to the N-terminus of MGDF. Such reaction

2167090

-46-

conditions generally provide for pK_a differences between the lysine amino groups and the α -amino group at the N-terminus (the pK_a being the pH at which 50% of the amino groups are protonated and 50% are not). The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired (i.e., the less reactive the N-terminal α -amino group, the more polymer needed to achieve optimal conditions). If the pH is higher, the polymer:protein ratio need not be as large (i.e., more reactive groups are available, so fewer polymer molecules are needed). For purposes of the present invention, the pH will generally fall within the range of 3-9, preferably 3-6.

Another important consideration is the molecular weight of the polymer. In general, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. Similarly, branching of the polymer should be taken into account when optimizing these parameters. Generally, the higher the molecular weight (or the more branches) the higher the polymer:protein ratio. In general, for the pegylation reactions contemplated herein, the preferred average molecular weight is about 2kDa to about 100kDa (the term "about" indicating ± 1 kDa). The preferred average molecular weight is about 5kDa to about 50kDa, particularly preferably about 12kDa to about 25kDa. The ratio of water-soluble polymer to MGDF protein will generally range from 1:1 to 100:1, preferably (for polypegylation) 1:1 to 20:1 and (for monopegylation) 1:1 to 5:1.

Using the conditions indicated above, reductive alkylation will provide for selective attachment of the polymer to any MGDF protein having an α -amino group at the amino terminus, and provide for a substantially homogenous preparation of monopolymer/MGDF

-47-

protein conjugate. The term "monopolymer/MGDF protein conjugate" is used here to mean a composition comprised of a single polymer molecule attached to an MGDF protein molecule. The monopolymer/MGDF protein conjugate
5 preferably will have a polymer molecule located at the N-terminus, but not on lysine amino side groups. The preparation will preferably be greater than 90% monopolymer/MGDF protein conjugate, and more preferably greater than 95% monopolymer MGDF protein conjugate,
10 with the remainder of observable molecules being unreacted (i.e., protein lacking the polymer moiety). The examples below provide for a preparation which is at least about 90% monopolymer/ protein conjugate, and about 10% unreacted protein. The monopolymer/protein
15 conjugate has biological activity.

For the present reductive alkylation, the reducing agent should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation.
20 Preferred reducing agents may be selected from the group consisting of sodium borohydride, sodium cyanoborohydride, dimethylamine borane, trimethylamine borane and pyridine borane. A particularly preferred reducing agent is sodium cyanoborohydride.

25 Other reaction parameters, such as solvent, reaction times, temperatures, etc., and means of purification of products, can be determined case-by-case based on the published information relating to derivatization of proteins with water soluble polymers
30 (see the publications cited herein). Exemplary details are shown in the Examples section below.

One may choose to prepare a mixture of polymer/protein conjugate molecules by acylation and/or alkylation methods, and the advantage provided herein is
35 that one may select the proportion of monopolymer/ protein conjugate to include in the mixture. Thus, if

2167090

-48-

desired, one may prepare a mixture of various protein
with various numbers of polymer molecules attached
(i.e., di-, tri-, tetra-, etc.) and combine with the
monopolymer/protein conjugate material prepared using
5 the present methods, and have a mixture with a
predetermined proportion of monopolymer/protein
conjugate.

The working examples below demonstrate the
preparation of chemically modified MGDF and the
10 preparation of MGDF pegylated via acylation and
alkylation. Thus, other aspects of the present
invention relate to these preparations.

Generally, conditions which may be alleviated
or modulated by administration of the present
15 polymer/MGDF include those described above for MGDF
molecules in general. However, the polymer/MGDF
molecules disclosed herein may have additional
activities, enhanced or reduced activities, or other
characteristics, as compared to the non-derivatized
20 molecules.

In yet another aspect of the present
invention, provided are pharmaceutical compositions of
the above chemically modified MGDF molecules. Such
pharmaceutical compositions may contain any of the
25 ingredients specified herein for non-derivatized MGDF
molecules.

As further studies are conducted, information
will emerge regarding appropriate dosage levels for
treatment of various conditions in various patients, and
30 the ordinary skilled worker, considering the therapeutic
context, age and general health of the recipient, will
be able to ascertain proper dosing. Generally, the
dosage will be between 0.01 $\mu\text{g/kg}$ body weight
(calculating the mass of the protein alone, without
35 chemical modification) and 300 $\mu\text{g/kg}$ (based on the
same). The preferred dose will generally be from 5

-49-

μg/kg body weight to 100 μg/kg body weight, particularly preferably from 10 μg/kg body weight to 75 μg/kg body weight.

5 The present invention also provides a method for producing MGDF (i.e., Mpl ligand) polypeptides or active fragments thereof. One method of the present invention involves introducing the cDNA encoding an Mpl
10 expression system for Mpl ligand. A selected host cell is transfected with the vector and cultured. The method of this present invention therefore comprises culturing a suitable cell or cell line, which has been transfected with a DNA sequence coding on expression for an Mpl
15 ligand polypeptide under the control of known regulatory sequences. Regulatory sequences include promoter fragments, terminator fragments and other suitable sequences which direct/control the expression of the protein in an appropriate host cell. The expressed
20 factor is then recovered, isolated and purified from the culture medium (or from the cell, if expressed intracellularly) by appropriate means known to one of skill in the art. Additionally, the methods disclosed in U.S. Patent 5,272,071 are also contemplated to be
25 applicable to the inventive polynucleotides/
polypeptides.

 Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells
30 and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, *Nature* **293**: 620-625 (1981), or alternatively, Kaufman et al., *Mol. Cell. Biol.*, **5** (7): 1750-1759 (1985) or Howley
35 et al., *U.S. Pat. No. 4,419,446*. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7

2167090

-50-

cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5 α , DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, *Streptomyces spp.*, and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al., *Genetic Engineering* 8: 277-298 (1986) and references cited therein.

The present invention also provides recombinant molecules or vectors for use in the method of expression of novel Mpl ligand polypeptides. These vectors contain the Mpl ligand DNA sequences and which alone or in combination with other sequences code for Mpl ligand polypeptides (with or without signal peptides) of the invention or active fragments thereof. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of Mpl ligand polypeptides. The vector employed in the method also

-51-

contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells.

5 One vector is pXM, which is particularly desirable for expression in COS cells [Y.C. Yang et al., *Cell* **47**: 3-10 (1986)]. Another vector which is desirable for expression in mammalian cells, e.g., CHO cells, is pEMC2B1. Mammalian cell expression vectors
10 described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors, e.g. replicons, selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known procedures. See,
15 Kaufman et al., *J. Mol. Biol.* **159**: 511-521 (1982); and Kaufman, *Proc. Natl. Acad. Sci. USA* **82**: 689-693 (1985). Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome [Lusky et al., *Cell* **36**: 391-401 (1984)] and be replicated in cell lines such
20 as C127 mouse cells as a stable episomal element. The transfection of these vectors into appropriate host cells can result in expression of the Mpl ligand polypeptides.

Other appropriate expression vectors of which
25 numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression can also be used for this purpose.

The conditions to be treated by the methods and compositions of the present invention are generally
30 those which involve an existing megakaryocyte/platelet deficiency or an expected megakaryocyte/platelet deficiency in the future (e.g., because of planned surgery). Such conditions will usually be the result of a deficiency (temporary or permanent) of active Mpl
35 ligand in vivo. The generic term for platelet deficiency is thrombocytopenia, and hence the methods

2167090

-52-

and compositions of the present invention are generally available for treating thrombocytopenia.

Thrombocytopenia (platelet deficiencies) may be present for various reasons, including chemotherapy and other therapy with a variety of drugs, radiation therapy, surgery, accidental blood loss, and other specific disease conditions. Exemplary specific disease conditions that involve thrombocytopenia and may be treated in accordance with this invention are: aplastic anemia, idiopathic thrombocytopenia, metastatic tumors which result in thrombocytopenia, systemic lupus erythematosus, splenomegaly, Fanconi's syndrome, vitamin B12 deficiency, folic acid deficiency, May-Hegglin anomaly, Wiskott-Aldrich syndrome, and paroxysmal nocturnal hemoglobinuria. Also, certain treatments for AIDS result in thrombocytopenia (e.g., AZT). Certain wound healing disorders might also benefit from an increase in platelet numbers.

With regard to anticipated platelet deficiencies, e.g., due to future surgery, an Mpl ligand of the present invention could be administered several days to several hours prior to the need for platelets. With regard to acute situations, e.g., accidental and massive blood loss, an Mpl ligand could be administered along with blood or purified platelets.

The Mpl ligands of this invention may also be useful in stimulating certain cell types other than megakaryocytes if such cells are found to express Mpl receptor. Conditions associated with such cells that express the Mpl receptor, which are responsive to stimulation by the Mpl ligand, are also within the scope of this invention.

MGDF molecules that are not themselves active in the activity assays presented herein may be useful as modulators (e.g., inhibitors or stimulants) of the Mpl receptors *in vitro* or *in vivo*.

2167090

-53-

The polypeptides of the present invention may also be employed alone, or in combination with other cytokines, soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies, in the treatment of the above-identified conditions.

Therefore, as yet another aspect of the invention are therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of an Mpl ligand polypeptide or a therapeutically effective fragment thereof in admixture with a pharmaceutically acceptable carrier. The carrier material may be water for injection, preferably supplemented with other materials common in solutions for administration to mammals. Typically, an Mpl ligand therapeutic will be administered in the form of a composition comprising purified protein in conjunction with one or more physiologically acceptable carriers, excipients, or diluents. Neutral buffered saline or saline mixed with serum albumin are exemplary appropriate carriers. Preferably, the product is formulated as a lyophilizate using appropriate excipients (e.g., sucrose). Other standard carriers, diluents, and excipients may be included as desired. Other exemplary compositions are Tris buffer, pH 8.0 and acetate buffer, pH 5.0, which, in each case, may further include sorbitol.

The present compositions can be systemically administered parenterally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such pharmaceutically acceptable protein solutions, with due regard to pH, isotonicity, stability and the like, is within the skill of the art.

2167090

-54-

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician, considering various factors which modify the action of drugs, e.g. the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 0.1-1000 micrograms of Mpl ligand protein or fragment thereof per kilogram of body weight.

The therapeutic methods, compositions and polypeptides of the present invention may also be employed, alone or in combination with other cytokines, soluble Mpl receptor, hematopoietic factors, interleukins, growth factors or antibodies in the treatment of disease states characterized by other symptoms as well as platelet deficiencies. It is anticipated that an Mpl ligand molecule will prove useful in treating some forms of thrombocytopenia in combination with general stimulators of hematopoiesis, such as IL-3 or GM-CSF. Other megakaryocytic stimulatory factors, i.e., meg-CSF, stem cell factor (SCF), leukemia inhibitory factor (LIF), oncostatin M (OSM), or other molecules with megakaryocyte stimulating activity may also be employed with Mpl ligand. Additional exemplary cytokines or hematopoietic factors for such co-administration include IL-1 alpha, IL-1 beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, colony stimulating factor-1 (CSF-1), GM-CSF, granulocyte colony stimulating factor (G-CSF), EPO, interferon-alpha (IFN-alpha), IFN-beta, or IFN-gamma. It may further be useful to administer, either simultaneously or sequentially, an effective amount of a soluble mammalian Mpl receptor, which appears to have an effect of causing megakaryocytes to fragment into platelets once the megakaryocytes have reached mature form. Thus,

2167090

-55-

administration of Mpl ligand (to enhance the number of mature megakaryocytes) followed by administration of the soluble Mpl receptor (to inactivate the ligand and allow the mature megakaryocytes to produce platelets) is
5 expected to be a particularly effective means of stimulating platelet production. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by
10 conventional methods.

Other uses for these novel polypeptides are in the development of antibodies generated by standard methods. Thus, antibodies that react with the Mpl ligands of the present invention, as well as reactive
15 fragments of such antibodies, are also contemplated. The antibodies may be polyclonal, monoclonal, recombinant, chimeric, single-chain and/or bispecific, etc. The antibody fragments may be any fragment that is reactive with the Mpl ligand of the present invention,
20 such as, Fab, Fab', etc. Also provided by this invention are the hybridomas generated by presenting Mpl ligand or a fragment thereof as an antigen to a selected mammal, followed by fusing cells (e.g., spleen cells) of the animal with certain cancer cells to create
25 immortalized cell lines by known techniques. The methods employed to generate such cell lines and antibodies directed against all or portions of a human Mpl ligand polypeptide of the present invention are also encompassed by this invention.

30 The antibodies may be used therapeutically, such as to inhibit binding of the Mpl ligand and its receptor. The antibodies may further be used for *in vivo* and *in vitro* diagnostic purposes, such as in labeled form to detect the presence of the Mpl ligand in
35 a body fluid.

2167090

-56-

* * *

The following examples are included to more fully illustrate the present invention. Additionally, these examples provide preferred embodiments of the invention, but are not meant to limit the scope thereof, unless so indicated. Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely recognized manuals of molecular biology such as, for example, Sambrook et al., *Molecular Cloning*, Second Edition, Cold Spring Harbor Laboratory Press (1987) and in Ausubel et al., (Eds), *Current Protocols in Molecular Biology*, Greene associates/Wiley Interscience, New York (1990).

EXAMPLE 1

Aplastic Canine Plasma

Heparinized aplastic canine plasma ("APK9") or normal canine plasma ("NK9") was produced as described in the following publications, except that 450 rads of total body irradiation were delivered to recipients:

1. Mazur, E. and South, K. *Exp. Hematol.* **13**:1164-1172 (1985).

2. Arriaga, M., South, K., Cohen, J.L., and Mazur, E.M. *Blood* **69**: 486-492 (1987).

3. Mazur, E., Basilico, D., Newton, J.L., Cohen, J.L., Charland, C., Sohl, P.A., and Narendran, A. *Blood* **76**: 1771-1782 (1990).

-57-

EXAMPLE 2

Human Megakaryocyte Assay

5 APK9 and fractionated APK9 were assayed for
the ability to stimulate development of human
megakaryocytes from CD34⁺ progenitor cells. CD34-
selected cells were obtained from peripheral blood cells
as described (Hokom, M.H., Choi, E., Nichol, J.L.,
10 Hornkohl, A., Arakawa, T., and Hunt, P. *Molecular
Biology of Haematopoiesis* 3:15-31, 1994) and were
incubated in the following culture medium: Iscove's
modified Dulbecco's medium (IMDM; GIBCO, Grand Island,
NY) supplemented with 1% Glutamine Pen-strep (Irvine
15 Scientific, Santa Ana, CA) and 10% heparinized,
platelet-poor, human AB plasma. Also included were 2-
mercaptoethanol (10^{-4} M), pyruvic acid (110 µg/ml),
cholesterol (7.8 µg/ml), adenosine, guanine, cytidine,
uridine, thymidine, 2-deoxycytosine, 2-deoxyadenosine,
20 2-deoxyguanosine (10 µg/ml each, Sigma); human
recombinant insulin (10 µg/ml), human transferrin (300
µg/ml), soybean lipids (1%, Boehringer Mannheim,
Indianapolis, IN); human recombinant basic fibroblast
growth factor (2 ng/ml, Genzyme, Cambridge, MA); human
25 recombinant epidermal growth factor (15 ng/ml),
platelet-derived growth factor (10 ng/ml, Amgen, Inc.,
Thousand Oaks, CA). CD34-selected cells were plated at
 2×10^5 /ml culture medium, 15 µl final volume, in wells of
Terasaki-style microtiter plates (Vanguard, Inc.,
30 Neptune, NJ). Cells were incubated at 37°C for 8 days
in humidified boxes in 5% CO₂ in air, fixed directly to
the culture wells with 1% glutaraldehyde, and incubated
with a monoclonal antibody cocktail (anti-GPIb, anti-
GPIIb, (Biodesign) and anti-GPIb (Dako, Carpinteria,
35 CA). The immune reaction was developed with a
streptavidin-beta-galactosidase detection system

2167090

-58-

(HistoMark^{*}, Kirkegaard and Perry). Megakaryocytes, identified by a blue color, were counted with an inverted phase microscope at 100X magnification. Results were presented as the average number of megakaryocytes per well +/- standard error of the mean (SEM). In some cases, data were presented in terms of "megakaryocyte units/ml" where the degree to which a given sample induced megakaryocyte development was normalized to the positive APK9 control for that experiment. One unit is defined as the amount of material that results in the same number of megakaryocytes as 1 ul of APK9 standard. Activity was accepted as due to MPL ligand if it could be blocked with 5-10 ug/ml MPL-X (soluble Mpl receptor).

APK9 has been demonstrated to contain factor(s) that stimulate human megakaryocyte development in this system. CD34-selected cells incubated with 10% NK9 for 8 days show a negligible number of blue-stained megakaryocytes, whereas CD34-selected cells incubated with 10% APK9 for 8 days show a very large number of blue-stained megakaryocytes.

FIG. 2 shows that increasing concentrations of Mpl-X added to the human megakaryocyte culture system increasingly block megakaryocyte development. At concentrations of Mpl-X greater than 5 ug/ml, inhibition is complete. In this experiment, CD34-selected cells were stimulated with 5% APK9. This demonstrates that an activity which interacts with Mpl-X (presumptive Mpl ligand) is necessary for human megakaryocyte development, and implies that the Mpl ligand is present in APK9 itself.

It has been further demonstrated herein that the Mpl ligand activity necessary for human megakaryocyte development is present in APK9. APK9 (135 ml) was diluted 6-fold into Iscove's media and applied to an Mpl-X affinity column. Unbound material (flow

*Trademark

-59-

through) was collected and concentrated to the original volume before assay. Bound material was eluted in 10 ml of 1 M NaCl, and 20% of the pool was diafiltered and concentrated 4-fold for assay. CD34-selected cells
5 incubated in media alone did not develop into megakaryocytes. Cells incubated in 5% APK9 (same pool as applied to column) developed into 48 +/- 8 megakaryocytes per well. Cells incubated in 10% of the unbound material did not develop into megakaryocytes.
10 Cells incubated in 10% of the elution pool developed into 120 +/- 44 megakaryocytes per well. Both the column load and the elution pool activities were substantially completely inhibited with 5 µg/ml Mpl-X in the assay.

15

EXAMPLE 3

Transfection of murine or human Mpl receptor into a
murine cell line

20

A. Murine Mpl Receptor

The full length murine Mpl receptor cDNA was subcloned into an expression vector containing a transcriptional promoter derived from the LTR of Moloney
25 Murine Sarcoma virus. 5 µg of this construct and 1 µg of the selectable marker plasmid pWLNeo (Stratagene) were co-electroporated into an IL-3 dependent murine cell line (32D, clone 23; Greenberger^{*} et al., PNAS
80:2931-2936 (1983)). Cells were cultured for 5 days to
30 recover from the procedure, then incubated in selection media including 800 ug/ml Geneticin^{*} (G418, Sigma) and 1 ng/ml murine IL-3. The surviving cells were then divided into pools of 2x10⁵ cells and cultured until a population grew out which could be further analyzed. Six
35 populations were tested for surface expression of Mpl receptor by FACS analysis using a polyclonal rabbit

*Trademark

2167090

-60-

antipeptide serum. One population was chosen for FACS sorting using the same antipeptide serum as before. Single-cell clones of the parent cell line were selected by growth in 10% APK9 and Geneticin*. After selection in
5 APK9 for 35 days, the cells were maintained in 1 ng/ml murine IL-3. One of the subclones, 1A6.1, was used for this body of work.

10 B. Human Mpl Receptor

The full length human Mpl receptor sequence (Vigon, I., et al., PNAS 89: 5640-5644 (1992)) was subcloned into an expression vector containing the transcriptional promoter of Moloney Murine Sarcoma virus
15 (same vector as with the murine receptor). Six µg of this construct and 6 µg of an amphotrophic retroviral packaging construct (Landau, N.R., Littman, D.R., J. Virology 66: 5110-5113 (1992)) were transfected into 3 x 10⁶ 293 cells using a CaPO₄ mammalian transfection kit
20 (Stratagene). The same cells were retransfected after 2 days and again after 4 days. The day after the last transfection the 293 cells were cocultivated with the IL-3 dependent murine cell line (32D, clone 23; Greenberger et al., PNAS 80: 2931-2936 (1983)). After
25 24 hours, the 32D cells were rescued and banded in a BSA gradient (Path-o-cyte*; Miles Inc.). Cells were expanded in 1 ng/ml murine IL-3 and then were selected for growth in 20% APK9. Cells were sorted for cell surface
30 antipeptide serum. These cells were subsequently used in the assays.

*Trademark

2167090

-61-

EXAMPLE 4

1A6.1 assay for Mpl ligand

5 1A6.1 cells were washed free of culture IL-3
and replated (1000 cells/15 μ l total vol/ well) in
Terasaki-style microtiter plates in alpha MEM (Gibco)
supplemented with 10% fetal calf serum (FCS), Geneticin*
10 (800 μ g/ml) and 1% pen/strep (Gibco) in 1:1 serial
dilutions of test samples. After 48 hours, the number
of viable cells per well was determined microscopically.
One unit of activity was defined as that amount of
activity that resulted in 200 viable cells per well.
Activity was defined as due to Mpl ligand if it could be
15 completely blocked by including 5-10 μ g/ml Mpl-X in the
assay. Mpl ligand activity in APK9 averaged 4400 +/-
539 units/ml of aplastic plasma. Unless otherwise
indicated, units of Mpl ligand activity are defined in
the 1A6.1 assay.

20 Assays with cells transfected with the human
Mpl receptor gene (Example 3B) were carried out in
essentially the same manner as with the 1A6.1 cells.

25

EXAMPLE 5

Demonstration that Mpl-ligand is present in aplastic
plasma or sera of mouse, dog, pig and human sources

30 Mpl ligand is present in the aplastic plasma
or sera from murine, canine, porcine and human sources
(Table 2). Plasma was collected from BDF1 mice pre-
irradiation and 12 days post-irradiation (500 rads).
Plasma was tested in the 1A6.1 assay where it
35 demonstrated 2000 units/ml activity that was
substantially completely inhibitable with Mpl-X (10

*Trademark

2167090

-62-

ug/ml). Irradiated mouse plasma was also positive in the human megakaryocyte assay where it displayed an activity of 1833 units/ml. Plasma was collected from dogs pre-irradiation and 10 days post-irradiation (450
5 rads). Plasma was tested in both the 1A6.1 assay and human megakaryocyte assays. Activity was detected and completely inhibited by Mpl-X (10 ug/ml) in both assays. Plasma was collected from pigs pre-irradiation and 10 days post-irradiation (650 rads). Plasma was tested in
10 both the 1A6.1 assay and the human megakaryocyte assays. In both assays it displayed Mpl ligand activity (inhibitable by 10 ug/ml Mpl-X) comparable to that found in aplastic canine plasma. Sera from aplastic humans was obtained. This material was collected from bone marrow
15 transplantation patients. The sera from 6 patients were assayed in the 1A6.1 assay where it showed an activity of 903 units/ml, 88% of which was due to Mpl ligand (inhibitable with 10 ug/ml Mpl-X). Sera from 14
20 aplastic patients has also been tested in the human megakaryocyte assay. As a group, they displayed substantial activity, 941 meg units/ml, which was completely inhibitable with 10 ug/ml Mpl-X. Murine IL-3 data is included to demonstrate the specificity of the
25 growth of the cell line, it is not blocked by 10 ug/ml Mpl-X.

30

35

-63-

Table 2

<u>Species</u>	<u>1A6.1 Cell Assay</u> <u>(units/ml)</u>		<u>Human Meg Assay</u> <u>(meg units/ml)</u>	
	<u>media</u>	<u>+ Mpl-X</u>	<u>media</u>	<u>+Mpl-X</u>
Normal mouse	0+/-0	0+/-0	0+/-0	0+/-0
Aplastic mouse	2000	0	1833	not done
Normal canine	0+/-0	0+/-0	0+/-0	0+/-0
Aplastic canine	4400+/-539	0+/-0	792+/-128	0+/-0
Normal porcine	0+/-0	0+/-0	0+/-0	0+/-1
Aplastic porcine	3866+/-1136	0+/-0	1284+/-182	10+/-10
Normal human	0+/-0	0+/-0	0+/-0	0+/-0
Aplastic human	903+/-64	114+/-33	941+/-178	0+/-0
murIL3	6000+/-565	6000+/-565	not done	not done

EXAMPLE 6

5

Mpl ligand stimulates 1A6.1 cell growth and human
megakaryocyte development

10 Mpl ligand (enriched at least about 100,000-
fold after lectin and affinity chromatography
procedures; see Example 7) stimulates the growth of the
1A6.1 cell line and the development of human
megakaryocytes from CD34-selected peripheral blood cells
in a dose-dependent manner. The activity responsible is
15 due to Mpl ligand as shown in FIGS. 2 and 3 since the
activities in both assays can be completely blocked with
Mpl-X.

It has also been shown by the inventors that
FACS purified peripheral blood CD34⁺ cells, when

2167090

-64-

incubated in Mpl ligand (100 units/ml for 9 days in this case), develop into phenotypically normal, mature megakaryocytes. This establishes that purified Mpl ligand has the same effect on megakaryocytes as does crude APK9. Furthermore, this experiment used purified CD34⁺ cells (100% CD34⁺) as opposed to CD34-selected cells which are generally only 30-50% CD34⁺.

10

EXAMPLE 7

Purification of Canine Mpl LigandI. Summary

15

Proteins (25 kd and 31 kd) that display activities predicted for a ligand for the Mpl receptor were purified. The proteins were purified from the plasma of irradiated dogs by a scheme employing wheat germ agglutinin (WGA) affinity chromatography, Mpl receptor affinity chromatography, anion exchange chromatography, gel filtration chromatography, and C4 reversed phase HPLC. See, FIG. 4 for an overview of this purification scheme. The 25 kd and 31 kd Mpl ligands have been highly purified to apparent homogeneity and have been determined to contain the amino acid sequences disclosed herein.

20

25

II. Methods

30

A. Clarification of plasma.

Frozen plasma (a total of 20 liters) from irradiated dogs (see Example 1) was thawed overnight at 4°C; thawing of larger bottles was initiated at room temperature for several hours before placement in the cold room. Insoluble material was removed by centrifugation for 6 hours at 11,000 xg. The plasma was

35

-65-

diluted with phosphate buffered saline, pH 7.3, containing 0.01% sodium azide (PBS/azide) and filtered through a 1.2 μ m filter. The clarification procedure typically resulted in an approximate two-fold dilution of the starting material.

B. Wheat Germ Agglutinin Affinity Chromatography.

All operations were carried out at 4°C. The clarified plasma (in two batches) was applied to a column of immobilized wheat germ agglutinin (1 liter, 10 x 12 cm, E Y Laboratories), equilibrated in PBS/azide. After sample application, unbound material was washed from the column with PBS/azide, followed by a wash with 0.5 M NaCl in 20 mM Tris-HCl, pH 8. Mpl ligand activity, bound by the WGA column, was eluted with 0.35 M N-acetylglucosamine (GlcNAc), 0.5 M NaCl, 20 mM Tris-HCl, pH 8. Mpl ligand activity could not be detected in the flow through or wash fractions.

C. Mpl-X receptor affinity chromatography.

The soluble murine Mpl receptor (Mpl-X) that was used corresponded to the entire extracellular domain of the Mpl receptor minus Trp at position 483 (See Vigon, et al, 8: 2607-2615 (1993)). In order to optimize binding of Mpl ligand to the Mpl-X receptor affinity column, the WGA elution pool was concentrated using a membrane ultrafilter (10,000 molecular weight cut off, YM-10, Amicon) and NaCl adjusted to 0.2 M by subsequent dilution. The concentrated WGA pool was applied to a 20 ml m-Mpl-X (murine Mpl soluble receptor)/CNBr activated Sepharose^{*} column (2.6 x 4.2 cm, 1.5 mg m-Mpl-X per ml of resin) at a flow rate 0.9 ml/min. The column was washed with 40 ml of PBS/azide at 1.5 ml/min, followed by a high salt wash (405 ml) with 10 mM Tris-HCl, 1 M NaCl, 1 mM CHAPS, pH 8.0. The

*Trademark

2167090

-66-

column was then eluted with 20 mM CAPS, 1 M NaCl, 5 mM CHAPS, pH 10.5. Appropriate fractions were collected. Tris was added to each fraction to neutralize the pH.

Both the SDS-PAGE and the absorbance at 280nm of the elution profile of an Mpl-X receptor affinity column reveal an early protein peak in fractions 1-4, while the majority of the Mpl ligand activity eluted after fraction 5.

10 D. Mono-Q Anion exchange chromatography.

The highest purity fractions from several Mpl-X receptor affinity columns were combined, concentrated, and diafiltered against 20 mM Tris-HCl, 5 mM CHAPS, pH 8.7 to a final volume of 58.5 ml. The protein concentration of the pool was estimated by absorbance at 280nm to be 0.12 mg/ml (approximately 7 mg total protein). The pool was loaded at 0.5 ml/min onto a Mono Q*HR 5/5 column (Pharmacia) equilibrated in 20 mM Tris-HCl, 5 mM CHAPS, pH 8.7. The column was eluted with a linear gradient to 0.36 M NaCl in the same buffer over 27 minutes. The column was then washed with a 6 minute gradient to 0.54 M NaCl, and finally with a step wash at 0.9 M NaCl. One ml fractions were collected.

The elution profile of the Mono Q* column shows that no Mpl ligand, and negligible protein, could be detected in the flow-through and wash fractions. Much of the Mpl ligand activity elutes in fractions 5-7, during the initial stages of the NaCl gradient. A "shoulder" of activity is observed in fractions 8-10, followed by a second major peak comprising fractions 11-15.

A distinct 25 kd band is observed by SDS-PAGE (nonreducing) in the active fractions. The intensity of the band directly corresponds with the Mpl ligand activity in the fractions. The band was absent in fractions 3 and 4 (no activity). It was prominent in

*Trademark

B

-67-

fractions 5 and 6 (1A6.1 activity peak) and a similar, intensely stained band, was present in fractions 11-14 (1A6.1 activity peak). The band is faint in the pool of fractions 15 and 16, corresponding with the significantly lower activity in fraction 16.

E. Gel Elution Experiments.

Gel elution experiments were performed using aliquots of Mono Q* fractions 5 and 6 or Mono Q* fractions 13 and 14. For these experiments, pools of fractions 5 and 6 (6 μ l each) or 13 and 14 (7.5 μ l each) were made, mixed with SDS-PAGE sample buffer (nonreducing), and applied to 12% SDS gels. Upon completion of electrophoresis, lanes of interest were sliced (1 mm) and the slices were diced into small pieces with razor blades. The pieces were transferred to 1.5 ml microfuge tubes containing 0.5 ml PBS/5mM CHAPS and gently agitated overnight at 4°C. The next day the tubes were spun briefly, an aliquot was removed, and the sample was diafiltered against Iscove's medium supplemented with BSA as a carrier protein. The diafiltered samples were submitted for assay.

The results reveal that two peaks of Mpl ligand activity can be observed. One peak corresponds to the 25 kd region of the gel, while a second peak of Mpl ligand activity is observed in the 31 kd region.

F. Superdex 200 Gel Filtration.

Fractions 13-16 from the Mono Q* anion exchange column, as well as two equivalent fractions from a second Mono Q* fractionation, were combined and concentrated using a membrane ultrafilter (Centricon-10*, Amicon). SDS was added to a final concentration of 0.1%, and the sample was injected onto a Superdex 200 HR* 10/30 (Pharmacia) column. The column was equilibrated in 50 mM Tris-HCl, 0.1% SDS, pH 7.5 at a flow rate of

*Trademark

-68-

0.3 ml/min, and was operated at room temperature. One minute fractions were collected. The results were that most of the protein in the sample elutes in fractions 32-40, while the Mpl ligand activity is detected in fractions 42-46. Analysis of fractions SDS-PAGE showed a distinct 25 kd band in the active fractions.

G. C4 Reversed Phase HPLC.

Superdex^{*} 200 fractions 43-46 combined or fraction 42 alone were concentrated using a membrane ultrafilter (Microcon-10^{*}, Amicon^{*}). The concentrated pools were separately applied to a 1 x 100 mm C4 reversed phase microbore column (SynChropak RP-4^{*}). The column was equilibrated in 0.04% TFA in water (A Buffer); B Buffer was 0.035% TFA in 80% acetonitrile. After injection of the sample, a linear gradient to 45% B over 4 min was performed, followed by a linear gradient to 75% B over 40 min. Flow rate was 75 µl/min. The results of purification of fraction 42 are presented in FIG. 5. Distinct Mpl ligand activity peaks were observed in fractions 21-23. These fractions were analyzed on a 14% polyacrylamide gel under nonreducing and reducing conditions. Fraction 21 was composed of a single 31 kd band; fraction 23 was composed of a single, broad 25 kd band; and fraction 22 contained bands in both the 25 kd and 31 kd regions. No other significant bands were visible. Note that earlier gel elution experiments had ascribed Mpl ligand activity to both of these regions. A single, minor high molecular weight band was observed in all fractions of the nonreducing gel, but could not be seen in the reducing gel.

*Trademark

-69-

H. N-terminal Sequence Analysis of 25 kd and 31 kd Mpl ligands.

N-terminal sequence analysis was carried out on C4 RP-HPLC fractions containing activity. The sequences determined for these proteins are reported above. In addition to the major sequence corresponding to the 25 kd band (at least 90% of the total of the applied sample), sequencing detected two minor sequences (which were associated with the minor contaminating band described in part G above). Comparisons with known sequences revealed that the minor sequences were canine Ig heavy chain and kappa chain. If desired, these minor impurities could be further reduced in quantity by application of another purification step, such as preferably another gel filtration step.

I. Comparison of Mpl ligand activities in the C4 purified fractions

FIG. 6 shows data demonstrating that the activities present in fractions 22 and 23 from the C4 RP-HPLC chromatography step are substantially equivalent. Fraction 22 contained a mixture of the 25 and 31 kd bands, whereas fraction 23 contained only the 25 kd band. Aliquots of each fraction were diluted 1:45000. The diluted fractions stimulated 1A6.1 cell growth substantially equally, (fraction 22, 5400 cells per well; fraction 23, 6000 cells per well). The diluted fractions were incubated with increasing concentrations of Mpl-X. The fractions were equally sensitive to inhibition by Mpl-X, both being completely blocked with 7-1.4 ug/ml. This indicates that the active protein(s) in each fraction are Mpl ligand species with equivalent biological activity.

2167090

-70-

EXAMPLE 8

Comparison of Mpl ligand to other factors on
megakaryocyte development

5

A number of recombinant factors or organic compounds such as phorbol myristic acetate (PMA) have been reported to impact megakaryocyte growth or development. Accordingly, the effects of these factors on CD34-selected peripheral blood cells were investigated. Human recombinant interleukin 3 (IL-3, 1-2 ng/ml), stem cell factor (SCF, 50 ng/ml), interleukin 6 (IL-6, 25 ng/ml), erythropoietin (EPO, 1 Unit/ml), leukemia inhibitory factor (LIF, 10 ng/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF, 25 ng/ml, Amgen, Inc.); interleukin 11 (IL-11, 25 ng/ml, R+D Systems, Minneapolis, MN); phorbol myristic acetate (PMA, 10^{-10} M, Sigma) were added to cultures as indicated. Mpl ligand was used at 275 units per ml, APK9 was used at 5% (equivalent to 220 units/ml). Factors tested in combination were at the same concentration as when tested individually. After 8 days in culture, the cells were fixed directly in the wells and stained for megakaryocytes (n=6 wells per condition) or counted for total cell number (n=3 wells per condition). Data are presented as mean +/- SEM.

FIG. 7 shows that APK9 and Mpl ligand resulted in the greatest number of megakaryocytes per well. IL-3 also resulted in megakaryocyte development, especially in combination with SCF. IL-6, IL-11, or EPO had little effect on megakaryocyte numbers either alone or in combination with IL-3. PMA, LIF and GM-CSF had little effect. In FIG. 8 are data from the same experiment showing the total number of cells found per well ("cellularity"). APK9 and Mpl ligand had little effect on cellularity while IL-3 and SCF had modest effects.

-71-

SCF and IL-3 in combination had the greatest effects. The data shown in FIGS. 7 and 8 were used to calculate percentages of megakaryocytes per well, as shown in FIG. 9. Clearly, the factor which results in the greatest percentage of megakaryocytes per culture well is Mpl ligand, the active ingredient in APK9. This is indicative of the specificity of Mpl ligand towards megakaryocytes.

EXAMPLE 9

The megakaryocyte promoting activity of Mpl ligand is not dependent on human IL-3

Mpl ligand stimulates the development of human megakaryocytes when used as a supplement to the culture medium described in Example 2. Although IL-3 is not an ingredient of the medium, it could be present in undetectably low levels in the normal human plasma present in the medium. However, even if present, IL-3 is not involved in Mpl ligand-induced megakaryopoiesis. This is shown in FIG. 10. IL-3 at 2 ng/ml contains an activity in the human meg assay of 14,900 meg units/ml. This activity is 97% inhibited with anti-IL-3 (3.3 ug/ml; Genzyme, Cambridge, MA). MPL ligand at 8203 meg units/ml was not inhibited with anti-IL-3.

EXAMPLE 10

Analysis of porcine Mpl ligand

I. Summary

Proteins from irradiated pig plasma with Mpl ligand activity were characterized with WGA affinity chromatography, Mpl receptor affinity chromatography,

-72-

ion exchange chromatography and C4 reverse phase HPLC. The activity was also characterized by elution from slices from SDS-polyacrylamide gels.

5

<u>Chromatography</u>	<u>Comments</u>
WGA affinity* column	4.4 x 10 ⁶ units applied. 3.4 x 10 ⁶ units recovered
Mpl receptor column	2.7 x 10 ⁶ units applied 2.4 x 10 ⁶ units recovered
Mono S* ion exchange pH 6.0	2.4 x 10 ⁶ units applied 4.4 x 10 ⁶ units recovered
C4 reverse phase HPLC *	Activity recovered fractions 23-25
Gel elution Experiments	Two activities clearly distinguished, one at approximately 18 kd, the other at approximately 28 kd.

*Trademark

2167090

-73-

EXAMPLE 11

Cloning of the Human Mpl-ligand, Human MGDF

5

Two approaches are outlined in the following:

I. First Exemplary Cloning Approach

10

A. Generation of human MDGF probe

A number of degenerate PCR primers were designed based on the amino terminus sequence of the canine protein. Different primer pairs were used to amplify the MGDF gene from the human genomic DNA. After
15 40 cycles of amplification, using the sense primers 5' GCN CCN CCN GCN TGY GA 3' (SEQ ID NO: 4), encoding the first five amino acids of the canine protein (SEQ ID NO: 1) and the antisense primer: 5' GCA RTG YAA CAC RTG NGA RTC 3' (SEQ ID NO: 5), encoding amino acids 16 to 21 of
20 SEQ ID NO: 1, the PCR product was run on a 2.0% agarose gel in TBE buffer.

The 63 bp band was cut out from the agarose gel and reamplified using the same primer set. The PCR product was cloned in the PCR II vector* (Invitrogen, San
25 Diego). A number of colonies were screened by DNA sequencing. The plasmid DNA encoding a peptide similar to the canine MGDF protein was used as the source to generate a radioactive probe to screen the cDNA libraries. The amino acid sequence encoded by the gene
30 fragment is as follows:

Ala-Pro-Pro-Ala-Cys-Asp-Leu-Arg-Val-Leu-Ser-Lys-Leu-
Leu-Arg-Asp-Ser-His-Val-Leu-His (SEQ ID NO: 6)

35

The agarose band containing the human MGDF was used to generate the probe by hot PCR. A typical PCR

*Trademark

B

2167090

-74-

reaction of 100 μ l contained the following ingredients:

	template DNA	2-3 μ l
	5' primer (SEQ ID NO:4)	1 μ l, 20 pmoles
5	3' primer (SEQ ID NO:5)	1 μ l, 20 pmoles
	10 X buffer	10 μ l
	dATP (0.1 mM)	2 μ l
	dTTP (10 mM)	2 μ l
	dGTP (10 mM)	2 μ l
10	dCTP (0.1mM)	2 μ l
	dCTP, p32 (10 uC/ μ l)	5 μ l
	dATP, p32 (10 uC/ μ l)	5 μ l
	Taq DNA polymerase	0.5 μ l, 2.5 units
	water	77 μ l
15		
	total volume	100 μ l

The amplification conditions were as follows:

20	initial heating	94°C, 2 min
	anneal	53°C, 30 sec
	extension	72°C, 30 sec
	denaturation	94°C, 30 sec.

25 40 cycles of amplification was carried out in a Perkin Elmer GeneAmp System 9600.*

The product was purified by passing through a push column (Stratagene, San Diego). One 1 μ l of the probe was counted in a scintillation counter. Probes
 30 containing 1 to 3 million counts per ml were added to the hybridization mix.

*Trademark

-75-

B. Construction of fetal liver library

Human fetal liver polyA⁺ RNA was purchased from Clontech laboratories. About 4 µg of RNA was used
5 for cDNA synthesis, in which priming was carried out using a random hexamer, 5' GTA CGC GTT CTA GAN NNN NNT 3', (SEQ ID NO: 7) attached to an oligo containing an Xba I site.

The Gibco-BRL protocol was used to generate
10 the double stranded cDNA. The Eco R I-Bst X I adaptor (Invitrogen, San Diego) was ligated to the double stranded cDNA, followed by digestion with the restriction enzyme, Xba I. Size selection of the cDNA was carried out on a S500 Sephacryl^{*} column (Life
15 Technologies, Inc.). cDNAs larger than 400 bp were ligated to the mammalian expression vector v19.8 (Martin, F., Cell 63: 203-211 (1990)) which was already digested with Eco RI and Xba I. Competent E. coli DH10 cells were transformed and the resulting cDNA library
20 was split into 7 pools of 100,000 cDNA each.

C. Screening the lambda library

A human fetal kidney library in lambda gt11^{*} was bought from Clontech with a titer of 650 million
25 pfu/ml. About 2 million plaques were screened with a probe generated by PCR (see above). Hybridization was done in 6 X SSC, 5 X Denhardt, 0.1% SDS, 100 µg/ml single strand salmon sperm DNA for 15 hours at 56°C.

Multiple rounds of screening were carried out.
30 DNA was amplified from single plaques and hybridized with the internal primer 5' AGT TTA CTG AGG ACT CGG AGG 3' (SEQ ID NO: 8) encoding amino acids 7 to 13 in SEQ ID NO: 6 to identify the true positives.

*Trademark

-76-

D. 3 prime Rapid Amplification of cDNA Ends
(RACE)

Polyadenylated RNA from human fetal kidney and fetal liver were bought from Clontech. One microgram RNA was reverse transcribed using the oligo 5' TTC GGC CGG ATA GGC CTT TTT TTT TTT TTT 3' (SEQ ID NO: 9) as the primer.

The Gibco-BRL cDNA synthesis kit (Life Technologies Inc., Cat. # 18267-013) was used to generate the first strand cDNA. The final volume was 30 μ l. The reaction was stopped by adding 500 mM EDTA to a final concentration of 10 mM and kept at -20°C.

For initial PCR, 0.5 μ l of cDNA was used as the template per reaction. The primer SEQ ID NO: 9 and the competitor oligo 5' TTC GGC CGG ATA GGC CTT TTT TTT TTT TT-P 3' (SEQ ID NO: 10) were used as the antisense primers, while the oligonucleotide 5' TGC GAC CTC CGA GTC CTC AG 3' (SEQ ID NO: 11) encoding amino acids 5 to 11 of SEQ ID NO: 6, was used as the sense primer. Forty cycles of amplification were carried out using the following protocol: 94°C, 30 sec; 65°C, 30 sec; 72°C, 30 sec, after an initial 2 min incubation at 94°C. A Perkin Elmer GeneAmp System 9600* was used for amplification.

Nesting was carried out using the sense primer 5' GAG TCC TCA GTA AAC TGC TTC GT 3' (SEQ ID NO: 12) encoding amino acids 8 to 14 of SEQ ID NO: 6, while SEQ ID NO: 9 and SEQ ID NO: 10 served as the antisense primers. Forty cycles of amplification were carried out with annealing at 65°C. The PCR products were run on a 0.8% agarose gel and then photographed under UV light. Bands around 0.8 to 1.2 kb were visible.

The PCR products were then cloned in the vector PCR II* (Invitrogen). Individual colonies were picked and plasmids were isolated using the Qiagen* kits

*Trademark

-77-

cat # 12143 and 12145. Double stranded dye primed sequencing was done using the vector primers. The sequences were analyzed by various types of GCG software.

5

E. 5' and 3' primer extension

In order to isolate the sequence of the full length MGDF gene, 3' and 5' primer extensions were carried out using different pools of fetal liver library as the template. For the amplification of the 5 primer of the cDNA, about 20 ng of cDNA from each pool was used as the template. A MGDF specific antisense primer 5' GGA GTC ACG AAG CAG TTT AC 3' (SEQ ID NO: 13) encoding amino acids 12 to 17 of SEQ ID NO: 6 and the 5' vector v19.8 sense primer 5' CCT TTA CTT CTA GGC CTG 3' (SEQ ID NO: 14) were used. Amplification was carried out for 30 cycles with annealing at 53°C. Nesting was done for 30 cycles with the antisense primers 5' GAG GTC ACA AGC AGG AGG A 3' (SEQ ID NO: 15) encoding amino acids 1 to 6 of SEQ ID NO: 6 and the vector primer SEQ ID NO: 14.

For the primer extension of the 3' ends of the MGDF cDNAs, the antisense vector primer 5' GGC ATA GTC CGG GAC GTC G 3' (SEQ ID NO: 16) and the MGDF specific primer 5' TCC TCC TGC TTG TGA CCT C 3' (SEQ ID NO: 17) encoding amino acids 1 to 6 of SEQ ID NO: 6, were used. Amplification was carried out for 30 cycles with annealing at 58°C.

Nesting amplification for 30 cycles was done using the MGDF primer SEQ ID NO: 12 and the vector primer SEQ ID NO: 16. Specific bands appeared in pool numbers 1, 7 and 8, which were cloned in the PCR II vector. Purified plasmid DNA from single colonies was purified and sequenced.

2167090

-78-

F. Isolation of full length clones of human MGDF

Many of the initial clones lacked part of the amino terminus of MGDF, since part of the MGDF sequence was used for priming and nesting. Primer 5' CCA GGA AGG ATT CAG GGG A 3' (SEQ ID NO: 18), whose sequence was obtained from the 5 primer extension experiments as described above was used as the sense primer. The vector primer SEQ ID NO: 16 served as the antisense primer. 35 cycles of amplification was carried out with annealing at 58°C. MGDF specific primer 5' CAA CAA GTC GAC CGC CAG CCA GAC ACC CCG 3' (SEQ ID NO: 19) with a Sal I site and the vector primer (SEQ ID NO: 15) were used for nesting for 35 cycles. The PCR product was cloned in PCR II* vector and sequenced.

II. Second Exemplary Cloning Approach

20 A. Cloning Of Canine MGDF N-Terminus cDNA

Degenerate oligonucleotide primers were designed based on the canine MGDF N-terminus amino acid sequence described in the previous section and used as primers in polymerase chain reactions (PCRs) to amplify MGDF-encoding cDNA sequences. Total RNA was prepared from canine kidney samples by the guanidinium isothiocyanate method of Chomzynski and Sacchi (Biochem. 162: 156-159 (1987)). First strand cDNA was prepared with a random primer-adapter 5' GGC CGG ATA GGC CAC TCN NNN NNT 3' (SEQ ID NO: 20) using MoMULV reverse transcriptase and used as template in subsequent PCRs.

PCR was performed on 0.5 microliters, about 50 ng, of the cDNA, using Primer A 5' GCN CCN CCN GCN TGY GA 3' (SEQ ID NO: 4), a sense strand primer encoding amino acids 1-6 of SEQ ID NO: 1, and either primer B 5' GCA RTG NAG NAC RTG NGA RTC 3' (SEQ ID NO: 5) or primer

*Trademark

B

-79-

C 5'GCA RTG YAA NAC RTG NGA RTC 3' (SEQ ID NO: 21),
which are antisense strand primers encoding amino acids
16-21 of SEQ ID NO: 1 with three extra nucleotides at
the 5' termini to increase annealing stability. PCR
5 with Taq polymerase was performed for 35 to 45 cycles,
until product bands were apparent on agarose gel
electrophoretic analysis. For the first two cycles of
PCR, the reannealing step was performed at 37°C for 2
minutes; for the remainder of the cycles reannealing was
10 at 50°C for 1 minute. Multiple product bands were
observed in each reaction. Portions of the gel
containing bands of approximately the expected size (66
bp) were collected with the tip of a Pasteur pipette and
re-amplified with the same primer pair. The DNA products
15 were cloned into vector PCR II (Invitrogen) according to
the manufacturer's instructions. Three clones were
sequenced and were found to encode, in one reading
frame, the expected canine MGDF sequence, residues 1-21.
In this way unique canine MGDF cDNA sequence was
20 obtained spanning the region from the third nucleotide
of codon 6 through the third nucleotide of codon 15.
One of these clones served as the template for
preparation of a labeled canine MGDF cDNA probe.

25 B. Construction of cDNA library from human
fetal liver

RNA has been isolated from human fetal Liver
(International Institute for the Advancement of
Medicine, Exton, PA) by lysis of tissue in 5.5 M
30 guanidinium thiocyanate and purification via CsTFA
(Pharmacia) centrifugation. Polyadenylated RNA was
selected using oligo (dT)₂₅ dynabeads* (Dynal, according
to manufacturer's instruction). Double stranded cDNA
was produced from this RNA using Superscript* plasmid
35 system for cDNA synthesis (Life Technologies, Inc.)
except a different linker adapter: 5' TTG GTG TGC ACT

*Trademark

B

2167090

-80-

TGT G 3' (SEQ ID NO: 22) and 5' CAC AAG TGC ACA CCA ACC
CC 3' (SEQ ID NO: 23), was used. After size selection
this cDNA was directionally inserted into the Bst XI and
Not I sites of the mammalian expression vector pBCB
5 (pBCB is derived from the plasmid Rc/CMV, Invitrogen,
comprising the puc19^{*} backbone, CMV promoter and BGH
polyadenylation site). The ligated DNA was
electroporated into electro competent bacterial strain
10B (Life Technologies, Inc.).

10

C. Screening of human fetal liver cDNA
library for MGDF

Filter replicas of the human fetal liver
library were hybridized to radioactively labeled canine
15 MGDF N-terminus cDNA PCR product (5x SSPE, 2x
Denhardt's, 0.05% Na pyrophosphate, 0.5% SDS, 100 µg/ml
yeast tRNA lysate and 100 µg/ml denatured salmon sperm
DNA) at 64°C for 18 h. Filters were washed at 64°C in
5x SSPE, 0.5% SDS and exposed over night. Two different
20 clones hybridizing to this probe were isolated and
analyzed.

D. Expression of human MGDF cDNA clones

Purified DNA from MGDF cDNA clones was transfected
25 into 293 EBNA cells (Invitrogen). 1.5 µg of DNA was
mixed with 7.5 ul Lipofectamine (Life Technologies,
Inc.) in 100 ul of serum free DMEM. After a 20 minute
incubation at room temperature the DNA-Lipofectamine
mixture was added to 5 x 10⁵ cells/well (24 well square
30 Greiner plate) in 400 ul DMEM, 1% serum (Fetal Clone
II) and incubated for 6 hours at 37°C. 500 ul DMEM,
20% serum (Fetal Clone II)^{*} was added to the cells. 16
hours later the media was aspirated and 500 ul DMEM, 1%
serum (Fetal Clone II)^{*} was added. 72 hours later the
35 conditioned media were collected and centrifuged
through a 0.22 micron spin-filter. The conditioned

*Trademark

B

-81-

media were assayed for MGDF biological activity.

III. Description and Activity of Human MGDF Clones

Based on the above-described cloning
5 strategies, the human cDNA clones shown in FIG. 11
(MGDF-1 and MGDF-2; SEQ ID NOS: 24, 25; and 26, 27) and
FIG. 12 (MGDF-3; SEQ ID NOS: 28, 29) were obtained.
Each of these sequences in the Figures contains a
putative signal sequence of amino acids 1-21, so the
10 mature proteins start at amino acid 22 in each case.

The results of activity assays using the cell-
based assay described in Example 4A above with MGDFs 1-3
are presented in Tables 3 and 4 below. In Table 3,
15 conditioned media from 293 EBNA cells transfected with
each construct was collected after 2 days of culture
then tested on 1A6.1 cells (32D/mur-MPL+) +/- 10 ug/ml
mur-MPL-X. In Table 4, conditioned media from 293 EBNA
cells transfected with each construct was collected
20 after 4 days of culture then tested on both 32D/mur-MPL+
cells (Example 3A) and 32D/hu-MPL+ cells (Example 3B).
As can be seen, human MGDF-1 and MGDF-2, but not MGDF-3,
were found to be active on cell lines expressing both
the murine and human forms of Mpl. The cell line
25 expressing the human MPL receptor is more responsive to
human MGDF-1 and MGDF-2 than is the cell line expressing
the murine Mpl receptor.

-82-

Table 3

<u>Clone</u>	<u>U/ml</u> <u>(- mur-MPL-X)</u>	<u>U/ml</u> <u>(+ mur-MPLX)</u>
Media	0	0
PBCO (control plasmid)	0	0
MGDF-1	12,800	800
MGDF-1 (repeat)	12,800	566
MGDF-2	4525	400
MGDF-2 (repeat)	12800	1131
MGDF-3	0	0
MGDF-3 (repeat)	0	0
APK9 control	4400+/-400	0

5

Table 4

<u>Clone</u>	<u>U/ml</u> <u>32D/mur-MPL+</u>	<u>U/ml</u> <u>32D/hu-MPL+</u>
MGDF-1	1600	25,600
MGDF-2	6400	50,000
MGDF-2 (repeat)	6400	50,000-100,000

10

The following Table 5 shows that the activities of human MGDF-1 and MGDF-2 on 32D/hu-MPL+ cells (Example 3B) are substantially completely inhibited by soluble human mpl receptor (hu-MPL-X). Hu-MPL-X was present as conditioned media collected from CHO cells producing the protein. The CHO hu-MPL-X conditioned media was concentrated 120-times then added to the cultures at 6.6%. Conditioned media from control

15

2167090

-83-

CHO cultures had no effect on the assay. The assay was carried out as described in Example 4B except that the viable cells were assessed after 3 days.

5

Table 5

<u>Clone</u>	U/ml <u>(-Hu-MPL-X)</u>	U/ml <u>(+Hu-MPL-X)</u>
MGDF-1	530	0
MGDF-2	270	0

Human Megakaryocyte Assay

10

MGDF-1 and MGDF-2 but not MGDF-3 induced the formation of megakaryocytes from peripheral blood CD34-selected cells. The experiment described in Table 6 was performed essentially as described in Example 2 except that peripheral blood cells were CD34-selected without elutriation and the culture was harvested after 7 days. Conditioned media from each 293 EBNA MGDF construct was used at 20% final volume +/- 30 ug/ml mur-MPL-X. APK9 control was used at 6% final volume.

15

-84-

Table 6

Clone	Megakaryocytes per Well <u>(-mur-MPL-X)</u>	Megakaryocytes per Well <u>(+mur-MPL-X)</u>
vector control	0	0
APK9 control	100 +/- 3	0
MGDF-1	142 +/- 48	17 +/- 2
MGDF-2	100 +/- 3	6 +/- 2
MGDF-2 repeat	86 +/- 10	0
MGDF-3	2 +/- 2	0

5

EXAMPLE 12

The following example describes the synthesis of 12 different pegylated MGDF molecules, PEG 9 - PEG 12 and PEG 14 - PEG 21. In each case, the MGDF molecule that was pegylated was *E. coli* derived MGDF-11 (amino acids 22-184, numbering from the beginning of the signal peptide or amino acids 1-163, numbering from the beginning of the mature protein). Details regarding all of these pegylated species are summarized in Tables 7-10 below.

12.1 Preparation of poly-MePEG-MGDF conjugates by MGDF acylation with activated MePEG derivatives

Preparation of poly-MePEG(20kDa)-MGDF conjugate (PEG 11).

A cooled (4°C) solution of MGDF (2.5 mg/ml) in 0.1 M BICINE* buffer, pH 8, was added to a 10-fold molar

*Trademark

B

-85-

excess of solid MePEG succinimidyl propionate (MW 20 kDa) (Shearwater Polymers, Inc.). The polymer was dissolved by gentle stirring and the reaction further conducted at room temperature.

5 The extent of the protein modification during the course of the reaction was monitored by size exclusion (SEC) HPLC using Superdex 200 HR 10/30* column (Pharmacia Biotech) eluted with 0.1 M sodium phosphate buffer pH 6.9 at 0.7 ml/min.

10 SEC HPLC analysis of the reaction mixture at the 30 minute time point indicated that no free protein was left in the reaction mixture. At this point the protein concentration in the reaction mixture was reduced to 1 mg/ml by addition of sterile water and the
15 pH of the mixture adjusted to 4 with several drops of 0.5M acetic acid.

 MePEG-MGDF conjugate was separated from the excess of MePEG and other reaction by-products by ion-exchange chromatography using SP Sepharose HP* (Pharmacia
20 Biotech) ion exchange resin.

 The reaction mixture was loaded (2.5 mg/ml of resin) onto the column and the unreacted MePEG was eluted with 3 column volumes of the starting buffer A (20 mM sodium phosphate, pH 7.2, 15% glycerol). After
25 that, the MePEG-MGDF conjugate was eluted using a linear gradient from 0% to 30% in 10 column volumes of the end buffer B (1M NaCl in buffer A). The eluent was monitored at 280 nm. Fractions containing poly-MePEG-MGDF conjugate were pooled, concentrated and sterile
30 filtered.

 The purified poly-MePEG-MGDF conjugate was analyzed by HPLC SEC using TSK-GEL G4000SWXL* and G2000SWXL* gel filtration columns coupled in series. Proteins were detected by UV absorbance at 280 nm. BIO-
35 RAD gel filtration standards served as globular protein

*Trademark

molecular weight markers.

As can be seen in FIG. 17A, HPLC SEC reveals two major components in the preparation (in about a 2 to 1 ratio) elution positions of which correspond to those of 370.9 kDa and 155.0 kDa globular proteins respectively. See also Table 8 below.

Conjugates PEG 9, PEG 10 and PEG 12 prepared by MGDF acylation with succinimidyl esters of MW=6-50 kDa MePEGs were conducted similarly. The major reaction parameters used in these preparations are summarized in Table 7.

Results of HPLC SEC analyses of these conjugates are shown in Table 8.

15

12.2. Preparation of poly-MePEG-MGDF conjugates by MGDF reductive alkylation with MePEG aldehydes.

Preparation of poly-MePEG(20kDa)-MGDF conjugate (PEG 20).

To a cooled (4°C), stirred solution of MGDF (2 ml, 2.5 mg/ml) in 100 mM sodium phosphate, pH 5, containing 20 mM NaCNBH₃ was added a 10-fold molar excess of monomethoxy-polyethylene glycol aldehyde (MePEG) (average molecular weight 20 kDa) and the stirring of the reaction mixture was continued at the same temperature.

The extent of the protein modification during the course of the reaction was monitored by SEC HPLC using Superdex 200 HR 10/30* column (Pharmacia Biotech) eluted with 0.1 M sodium phosphate buffer pH 6.9 at 0.7 ml/min.

After 16 hours the SEC HPLC analysis indicated that more than 90% of the initial amount of the protein has been modified. At this time the protein concentration in the reaction mixture was brought to 1

*Trademark

2167090

-87-

mg/ml by dilution of the reaction mixture with sterile water and the pH adjusted to 4 (0.5M acetic acid).

MePEG-MGDF conjugate was separated from the excess of MePEG and other reaction by-products by ion-exchange chromatography using SP Sepharose HP* (Pharmacia Biotech) ion exchange resin.

The reaction mixture was loaded (2.5 mg/ml of resin) onto the column and the unreacted MePEG was eluted with 3 column volumes of the starting buffer A (20 mM sodium phosphate, pH 7.2, 15% glycerol). After that, the MePEG-MGDF conjugate was eluted using a linear gradient from 0% to 30% in 10 column volumes of the end buffer B (1M NaCl in buffer A). The eluent was monitored at 280 nm. Fractions containing poly-MePEG-MGDF conjugate were pooled, concentrated and sterile filtered.

The purified poly-MePEG-MGDF conjugate was analyzed by HPLC SEC using TSK-GEL G4000SWXL* and G2000SWXL* gel filtration columns coupled in series. Proteins were detected by UV absorbance at 280 nm. BIO-RAD gel filtration standards served as globular protein molecular weight markers.

As can be seen in FIG. 17B, HPLC SEC reveals two major components (constituting 52% and 47% of the total amount) in the preparation, elution positions of which correspond to those of 359.4 kDa and 159.3 kDa globular proteins respectively. See also Table 8.

Conjugates PEG 18, PEG 19 and PEG 21 prepared by MGDF reductive alkylation with MePEG aldehydes of MW=6-25 kDa were conducted similarly. The major reaction parameters used in these preparations are summarized in Table 7.

Results of HPLC SEC analyses of these conjugates are shown in Table 8.

*Trademark

-88-

12.3. Preparation of monomethoxy-polyethylene glycol-MGDF conjugates with the site of attachment at the N-terminal α -amino residue.

5

Preparation of mono-MePEG (20kDa)-MGDF conjugate (PEG 16).

To a cooled (4° C), stirred solution of MGDF (2 ml, 2.5 mg/ml) in 100 mM sodium phosphate, pH 5, containing 20 mM NaCNBH₃ was added a 5-fold molar excess of methoxypolyethylene glycol aldehyde (MePEG) (average molecular weight 20 kDa) and the stirring of the reaction mixture was continued at the same temperature.

The extent of the protein modification during the course of the reaction was monitored by SEC HPLC using Superdex 200 HR 10/30 column (Pharmacia Biotech) eluted with 0.1 M sodium phosphate buffer pH 6.9 at 0.7 ml/min.

After 16 hours the SEC HPLC analysis indicated that about 90% of the initial amount of the protein has been modified. At this time the protein concentration in the reaction mixture was reduced to 1 mg/ml by dilution with sterile water and the pH of the reaction mixture adjusted to 4 (0.5 M acetic acid).

The mono-MePEG (20kDa)-MGDF conjugate was separated from the excess of MePEG and other reaction by-products by ion-exchange chromatography using SP Sepharose HP* (Pharmacia Biotech) ion exchange resin.

The reaction mixture was loaded (2.5 mg/ml of resin) onto the column and the unreacted MePEG was eluted with 3 column volumes of the starting buffer A (20 mM sodium phosphate, pH 7.2, 15% glycerol). After that, the MePEG-MGDF conjugate was eluted using a linear gradient from 0% to 25% of the end buffer B (1M NaCl in buffer A) in 20 column volumes. The eluent was monitored at 280 nm. Fractions containing poly-MePEG-

*Trademark

B

-89-

MGDF conjugate were pooled, concentrated and sterile filtered.

The homogeneity of the mono-MePEG-MGDF conjugates was determined by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis using 4-20% precast
5 gradient gels (NOVEX). One major band corresponding to the position of a 46.9 kDa protein was revealed.

The purified poly-MePEG-MGDF conjugate was analyzed by HPLC SEC using TSK-GEL G4000SWXL* and
10 G2000SWXL* gel filtration columns coupled in series. Proteins were detected by UV absorbance at 280 nm. The BIO-RAD gel filtration standards served as globular protein molecular weight markers.

As can be seen in FIG. 17C, SEC HPLC reveals
15 one major component in the preparation, elution positions of which corresponds to that of 181.1 kDa globular protein. See also Table 9.

Mono-MePEG-MGDF conjugates PEG 14, PEG 15 and PEG 17 prepared by MGDF reductive alkylation with MePEG
20 aldehydes of MW=6-25 kDa were conducted similarly. The major reaction parameters used in these preparations are summarized in Table 7.

Results of HPLC SEC analyses of these conjugates are shown in Table 9.

*Trademark

2167090

-90-

Table 7

Summary of MGDF modification reaction parameters

Code	Reactive MePEG		Reaction conditions				
	Type	MW	MGDF conc. mg/ml	pH	Temperature, °C	Time, h	Molar Ratio MePEG/ MGDF
PEG 9	NHS ester	6kDa	2.5	8	r.t.	0.5	15
PEG 10	NHS ester	6kDa	2.5	8	r.t.	0.5	10
PEG 11	NHS ester	20kDa	2.5	8	r.t.	0.5	10
PEG 12	NHS ester	50kDa	2.5	8	r.t.	0.5	5
PEG 14	ALDEHY DE	6 kDa	2.5	5	4°C	16	5
PEG 15	ALDEHY DE	12kDa	2.5	5	4°C	16	5
PEG 16	ALDEHY DE	20kDa	2.5	5	4°C	16	5
PEG 17	ALDEHY DE	25kDa	2.5	5	4°C	16	10
PEG 18	ALDEHY DE	6kDa	5	5	4°C	16	10
PEG 19	ALDEHY DE	12kDa	5	5	4°C	16	10
PEG 20	ALDEHY DE	20kDa	5	5	4°C	16	10
PEG 21	ALDEHY DE	25kDa	5	5	4°C	16	10

2167090

-91-

Table 8

Summary of poly-MePEG-MGDF characteristics by SEC HPLC

5

Code	Reactive MePEG	Apparent MW by SEC, kDa	Component amount, %
PEG 9	NHS	6kDa	87.9
	ester	52.7	75
PEG 10	NHS	6kDa	69.2
	ester	42.9	25 (shoulder)
PEG 11	NHS	20kDa	370.9
	ester	155.0	14 (shoulder)
PEG 12	NHS	50kDa	865.6
	ester	368.0	86
PEG 18	ALDEHY	6kDa	84.6
	DE	41.5	68
PEG 19	ALDEHY	12kDa	218.4
	DE	106.7	32
PEG 20	ALDEHY	20kDa	359.4
	DE	159.3	53
PEG 21	ALDEHY	25kDa	450.5
	DE	218.4	47

2167090

-92-

Table 9

Apparent molecular weights of mono-MePEG-MGDF Conjugates

5

Code	Reactive MePEG	Apparent MW by SEC, kDa	Apparent MW by SDS PAGE, kDa
	Type	MW	
PEG 14	ALDEHY DE	6 kDa	44.5 27.7
PEG 15	ALDEHY DE	12kDa	104.7 38.3
PEG 16	ALDEHY DE	20kDa	181.1 46.9
PEG 17	ALDEHY DE	25kDa	226.4 55.5

10 12.4. Preparation of DiMePEG (12kDa)-MGDF conjugates by reductive alkylation of MGDF with methoxy poly(ethylene glycol) aldehyde (PEG 22).

The following procedure results in a purified molecule referred to herein as PEG 22.

15 A 5-fold excess of methoxy polyethylene glycol aldehyde (MePEG; i.e., $\text{OHC}-(\text{CH}_2)_2\text{O}-(\text{CH}_2-\text{CH}_2\text{O})_n-\text{CH}_3$; where n = a repeat such that the molecular weight is ca. 12 kDa) (Shearwater Polymers), was added to a 2.5 mg/mL solution of MGDF (*E. coli* derived, 1 - 163) in 100 mM sodium acetate, pH 5.0 held at 5 degrees Celsius. After
20 mixing for 10 minutes, sufficient sodium cyanoboro-
hydride (Aldrich) was added to achieve a 20 mM concentration in the reaction mixture.

This mixture was stirred for 16 hours at

-93-

approximately 5 degrees C. At the end of this time, sufficient purified water, USP was added to bring the concentration of MGDF to 1 mg/mL. This was filtered through a 0.2 micron vacuum filter. 90 mg of reaction product were prepared in this manner. Small amounts of 1.0 M monobasic phosphate and 1 N sodium hydroxide solutions were added to the reaction product mixture to achieve a 10 mM phosphate, pH 6.8 solution.

The conjugate was purified on a cation exchange column. A 40mL SP-Sepharose High Performance* column was prepared with a bed height of 7.5 cm. The column was equilibrated with equilibration buffer (10 mM phosphate, pH 6.8, with 15% glycerol). The column was loaded at 2.2 mg/mL resin at 0.15 column volumes (CV) per minute. This was followed by a wash with the equilibration buffer until baseline was achieved. The column was eluted with a 10 column volume linear gradient from Buffer A (20 mM phosphate, pH 7.2 with 15 % glycerol) to Buffer B (Buffer A plus 0.3 M NaCl). The flow rate was maintained throughout at 0.15 CV per minute. The eluent was monitored at 280 nm.

SDS-PAGE gels were run of the fractions and those containing the DiPEG conjugate were pooled and filtered through a 0.2 micron unit.

25

EXAMPLE 13

Biological Activity of Pegylated MGDF Molecules

30

A. PEG-9 - PEG-12 and PEG-14 - PEG-21

Platelet counts from mice treated with recombinant human MGDF were measured and the results are presented in FIG. 18. CHO-derived 22-353 (open diamond), unpegylated *E. coli* 22-184 (open circles) and

*Trademark

2167090

-94-

pegylated *E. coli* 22-184 (closed circles) MGDF at the concentrations indicated in the description of the figures above, were injected subcutaneously into normal Balb/c mice once daily for 5 days. Test bleeds from a small lateral cut in a tail vein were collected 24 hours after the last injection. Blood cell analyses were performed with a Sysmex* electronic blood cell analyser (Baxter Diagnostics, Inc. Irvine, CA). Data are represented as the mean of determinations of 4 animals, +/- standard error of the mean. Other blood cell parameters such as total white blood cell counts or red blood cell counts were not affected by this treatment.

Additional forms of recombinant human MGDF were tested as above. Platelet counts from mice treated with either 50 ug/kg/day or 10 ug/kg/day of the indicated form of r-HuMGDF are shown in the following Table 10. Data are the mean of 4 animals and the standard errors are italicized.

*Trademark

-95-

Table 10

	<u>50 ug/kg/day</u>		<u>10 ug/kg/day</u>	
<u>Form</u>	<u>Mean (n=4)</u>	<u>sem</u>	<u>Mean (n=4)</u>	<u>sem</u>
CHO 22-353	4343	309	2571	80
E. coli 22-184	2021	29	1439	18
PEG 9	2728	56	2369	34
PEG 10 ^{NO}	2431	291	1556	126
PEG 11	3778	59	1861	73
PEG 12	3885	156	1740	88
PEG 14	3567	80	2020	63
PEG 15	4402	57	2834	99
PEG 16	4511	239	3215	11
PEG 17	4140	188	3113	261
PEG 18	4586	59	2931	129
PEG 19	3980	330	4189	80
PEG 20	3942	285	3054	339
PEG 21	4195	145	4002	91
Baseline	939	25		

Key to Table 10

In each of the following, the MGDF molecule that was
 pegylated was *E. coli* derived MGDF-11 (amino acids 22-
 184, numbering from the beginning of the signal peptide
 or amino acids 1-163, numbering from the beginning of
 the mature protein), as described in the above Example
 12:

-96-

	Name	Pegylation	Avg. MW of PEG	Reactive PEG molecule for synthesis
5	PEG 9	polypegylated	6 kDa	NHS ester of MePEG
	PEG 10	polypegylated	6 kDa	NHS ester of MePEG
	PEG 11	polypegylated	20 kDa	NHS ester of MePEG
	PEG 12	polypegylated	50 kDa	NHS ester of MePEG
10	PEG 14	monopegylated	6 kDa	Aldehyde of MePEG
	PEG 15	monopegylated	12 kDa	Aldehyde of MePEG
	PEG 16	monopegylated	20 kDa	Aldehyde of MePEG
	PEG 17	monopegylated	25 kDa	Aldehyde of MePEG
15	PEG 18	polypegylated	6 kDa	Aldehyde of MePEG
	PEG 19	polypegylated	12 kDa	Aldehyde of MePEG
	PEG 20	polypegylated	20 kDa	Aldehyde of MePEG
	PEG 21	polypegylated	25 kDa	Aldehyde of MePEG

20 The baseline counts are in normal animals
without administration of any materials.

 It is clear that pegylation of recombinant
human MGDF does not adversely affect the ability of the
molecule to increase platelet counts in recipient
25 animals, and may in fact increase the activity of the *E.*
coli product 22-184 to be as great or greater than that
seen with the CHO-derived 22-353 molecule.

B. PEG-22

30

 Results with PEG-22 are presented in FIG. 24.
Notably, normalization of platelet counts with PEG-22
occurred several days sooner than with full-length CHO
derived MGDF, PEG-16, or PEG-17.

35

-97-

EXAMPLE 14

Expression of recombinant human MGDF [1-163] in *E. coli*

5 To express r-HuMGDF in *E.coli*, the sequence encoding the first 163 amino acids of the mature protein was chemically synthesized utilizing optimal *E.coli* codons. Additionally, DNA sequences encoding the amino acids Methionine and Lysine were added to the 5' end of
10 the gene. Therefore, the r-HuMGDF protein encoded by this sequence is 165 amino acids in length beginning with Met-Lys. The sequence of this gene is set forth in FIG. 25.

 The synthesis of the r-HuMGDF (1-163) gene was
15 accomplished in several steps. First, complementary oligonucleotides (60-70bp in length) representing adjoining fragments of the gene were chemically synthesized utilizing optimal *E.coli* codons. During this synthesis, codons for the amino acids Methionine
20 and Lysine were placed at the 5' end of the mature gene, and a stop codon was placed at the 3' end of the gene. In addition, cutting sites for the restriction enzymes *Xba*I and *Hind*III were placed at the extreme 5' and 3' ends of the gene respectively, and a synthetic ribosome
25 binding site was placed an appropriate distance upstream of the initiating Methionine. Second, the complementary oligonucleotides for each gene fragment were annealed. Third, these individual synthetic gene fragments were amplified using the Polymerase Chain Reaction. Fourth,
30 amplified fragments were then sub-cloned into an appropriate vector. Fifth, the sequences of the sub-cloned fragments were verified. Sixth, the individual fragments were ligated together and sub-cloned into an appropriate vector, reconstructing the full-length r-
35 HuMGDF (1-163) gene. Finally, the sequence of the reconstructed gene was verified.

-98-

The synthetic r-HuMGDF gene fragment, flanked by *Xba*I and *Hind*III restriction sites at the 5' and 3' ends respectively, contains a ribosome binding site, the ATG start codon, the sequence encoding the mature Met-Lys r-HuMGDF protein, and the stop codon.

The above fragment was cloned into the *Xba*I and *Hind*III sites of the lactose-inducible expression vector pAMG11. The pAMG11 vector is a low-copy-number plasmid with a pR100-derived origin of replication. The expression plasmid pAMG11 can be derived from the plasmid pCFM1656 (ATCC# 69576, deposited February 24, 1994) by making a series of site directed base changes by PCR overlapping oligo mutagenesis. Starting with the *Bgl*II site (plasmid bp # 180) immediately 5' to the plasmid replication promoter *P*_{copB} and proceeding toward the plasmid replication genes, the base pair changes are as follows:

-99-

	<u>pAMG11 bp #</u>	<u>bp in pCFM1656</u>	<u>bp changed to in pAMG11</u>
	# 204	T/A	C/G
	# 428	A/T	G/C
5	# 509	G/C	A/T
	# 617	- -	insert two G/C bp
	# 679	G/C	T/A
	# 980	T/A	C/G
	# 994	G/C	A/T
10	# 1004	A/T	C/G
	# 1007	C/G	T/A
	# 1028	A/T	T/A
	# 1047	C/G	T/A
	# 1178	G/C	T/A
15	# 1466	G/C	T/A
	# 2028	G/C	bp deletion
	# 2187	C/G	T/A
	# 2480	A/T	T/A
20	# 2499-2502	<u>AGTG</u> TCAC	<u>GTCA</u> CAGT
	# 2642	<u>TCCGAGC</u> AGGCTCG	bp deletion
25	# 3435	G/C	A/T
	# 3446	G/C	A/T
	# 3643	A/T	T/A
30	# 4489-4512	- -	insert bps <u>GAGCTCACTAGTGTCTGACCTGCAG</u> CTCGAGTGATCACAGCTGGACGTC

(SEQ ID NOS: 30, 31)

and by substituting the DNA sequence between the unique
 35 AatII and ClaI restriction sites with the following
 oligonucleotide:

2167090

-100-

AatII (#4358)

5' CTCATAATTTTAAAAAATTCATTTGACAAATGCTAAAATTCTT-
3' TGCAGAGTATTAAAAATTTTAAAGTAAACTGTTTACGATTTTAAGAA-

5

-GATTAATATTCTCAATTGTGAGCGCTCACAATTTAT 3'
-CTAATTATAAGAGTTAACACTCGCGAGTGTTAAATAGC 5'

ClaI (#4438)

(SEQ ID NOS: 32, 33)

10

Expression of r-HuMGDF, cloned into pAMG11, is driven by a synthetic lactose-inducible promoter, such as Ps4, which has the following sequence:

15

5' GACGTCTCATAATTTTAAAAAATTCATTTGACAAATGCTAAA-
-ATTCTTGATTAATATTCTCAATTGTGAGCGCTCACAATTTATCGAT 3'.

(SEQ ID NO: 34)

20

The Ps4 promoter is repressed by the lactose repressor (LacI), the product of the *E.coli lacI* gene.

The pAMG11-r-HuMGDF plasmid was subsequently transformed into an *E.coli* K-12 strain containing the
25 *lacI^q* allele. The *lacI^q* allele is a mutation within the *lacI* promoter which increases the expression of LacI, and results in a more stringent control of protein expression from the Ps4 promoter. Therefore, in this strain, in the absence of lactose, expression of r-
30 HuMGDF is repressed by LacI. Upon the addition of lactose, LacI protein binding to the operator site on the Ps4 promoter is reduced, and transcription of r-HuMGDF from Ps4 is initiated. The *E. coli* host cell employed in this example is deposited under ATCC #69717,
35 as of November 30, 1994.

The *E. coli* host ATCC # 69717 was transformed

-101-

with the pAMG11-r-HuMGDF plasmid and was grown according to the following fermentation description. The *E. coli* strain is inoculated into Luria broth and then incubated at 30°C for approximately 12 hours. The cells are then

5 aseptically transferred into a fermentor that contains the batch medium (20 g/L yeast extract; 3.4 g/L citric acid; 15 g/L K₂HPO₄; 15 ml Dow P2000^{*}; 5 g/L glucose; 1 g/L MgSO₄·7H₂O; 5.5 ml/L trace metals; 5.5 ml/L vitamins). The batch phase of the process continues

10 until the culture reaches an optical density of 5.0 ± 1.0 at 600 nm. The fed-batch phase is then begun with the initiation of the first feed medium (700 g/L glucose; 6.75 g/L MgSO₄·7H₂O). The feed rate is adjusted every 2 hours per an established schedule. The

15 initiation of the second feed medium (129 g/L trypticase peptone; 258 g/L yeast extract) begins when the culture reaches an optical density of 20-25 at 600 nm. The second feed medium is maintained at a constant flow rate while the first feed medium continues to be adjusted.

20 The temperature during the entire fermentation is maintained at approximately 30°C. The culture is maintained at about pH 7 with the addition of acid and base as necessary. The desired dissolved oxygen level is maintained by adjusting the agitation and air-input

25 and oxygen-input rates in the fermentor. When the optical density of the culture reaches 57-63 at 600 nm addition of the third feed medium is initiated. The third feed medium (300 g/L lactose) is introduced to the fermentor at a constant flow rate; addition of the first

30 feed medium is discontinued and the second feed medium flow rate is changed to a new constant rate. The fermentation lasts approximately ten hours after initiation of the third feed medium. At the end of the fermentation, the culture is chilled to 15 ± 5°C. The

35 cells are harvested by centrifugation. The resulting paste is packaged and stored at < -60°C.

*Trademark

-102-

Purification of recombinant MGDF produced in *E. coli* as described above was carried out as follows. One thousand eight hundred grams of cell paste was suspended in about 18 liters of 10 mM EDTA and passed through a high pressure homogenizer at 15,000 psi. The broken cell suspension was centrifuged and the pellet was resuspended in 10 liter of 10 mM EDTA. The suspension was centrifuged and 200 g pellet was solubilized in 2 liter of 10 mM Tris, 8M Guanidine hydrochloride, 10 mM DTT, 5 mM EDTA, pH 8.7. This solution was slowly diluted into 200 liters of 10 mM CAPS, 3 M urea, 30% glycerol, 3mM cystamine, 1mM cysteine, pH 10.5.

The diluted solution was stirred slowly for 16 hr at room temperature and the pH was adjusted to 6.8. The pH adjusted solution was clarified and applied to a 2 liter CM Sepharose* column equilibrated with 10 mM sodium phosphate, 1.5 M urea, 15% glycerol, pH 6.8. After loading, the column was washed with 10 mM sodium phosphate, 15% glycerol, pH 7.2. MGDF was eluted with a gradient of 0 to 0.5 M sodium chloride, 10 mM sodium phosphate, pH 7.2.

The CM eluate was concentrated and buffer exchanged with 10 mM sodium phosphate pH 6.5 with a 10,000 molecular weight cut off membrane. The concentrated solution, at about 2 mg per ml, was treated with cathepsin C (500 to 1 molar ratio) for 90 minutes at ambient temperature.

The solution was then loaded to a 1.2 liter SP High Performance Sepharose* column equilibrated with 10 mM sodium phosphate, 15% glycerol, pH 7.2. After loading, MGDF was eluted with a gradient of 0.1 to 0.25 M sodium chloride, 10 mM sodium phosphate pH 7.2.

35

*Trademark

2167090

-103-

Ammonium sulfate was added to 0.6 M to the eluate from the SP High Performance column. The eluate was loaded to a 1.6 liter Phenyl Toyopearl* column equilibrated with 10 mM sodium phosphate, 0.6 M ammonium sulfate, pH 7.2. The MGDF peak was eluted with a gradient of 0.6 to 0 M ammonium sulfate, 10 mM sodium phosphate, pH 7.2.

The Phenyl Toyopearl* eluate was concentrated and buffer exchanged with a 10,000 molecular weight cut off membrane into 10 mM Tris, 5% sorbitol, pH 7.5.

EXAMPLE 15

In vivo, biological properties of r-HuMGDF (E. coli 1-163)

r-HuMGDF (E. coli 1-163), prepared as described in Example 14 above, was evaluated in rodents for biological efficacy. Normal, female Balb/c mice where injected subcutaneously for 5 consecutive days with increasing doses of r-HuMGDF. Doses ranged from 15 ug/kg/day to 1500 ug/kg/day. Twenty four hours after the last injection, blood cell counts were measured using an electronic cell counter (Sysmex, Baxter). A linear increase in platelet counts was observed with logarithmically increasing concentrations of the cytokine. Platelet counts increased to 300% of baseline values with 1500 ug/kg/day in this system. Other blood cell parameters were not affected with this treatment, such as white or red blood cell counts, or hematocrit.

Platelets were harvested from rats injected subcutaneously with 300 ug/kg/day r-HuMGDF (E. coli 1-163) for 6 days and evaluated for the ability to aggregate in response to ADP. The data indicate that platelets from treated animals are virtually indistinguishable from platelets from control animals in

*Trademark

B

-104-

that both populations are equivalently sensitive to the platelet agonist, ADP.

r-HuMGDF was also evaluated for the ability to abrogate the thrombocytopenia associated with chemotherapy and/or irradiation. Carboplatin, a chemotherapeutic which causes profound thrombocytopenia in humans, was used in these studies. Balb/c mice were injected subcutaneously with 1.25 mg carboplatin at the start of the study. After 24 hours, mice were injected daily with 100 ug/kg/day r-HuMGDF (*E. coli* 1-163), or excipient, for the remainder of the study. By Day 9, platelet counts dropped to roughly 15% of normal in excipient-treated mice but remained at baseline levels in mice treated with r-HuMGDF (see FIG. 20). For the irradiation studies, mice were subjected to a single dose of 500 rads of gamma-irradiation (Cesium source). This is a sublethal dose which results in a 90% reduction of platelet counts by Day 11. Platelet counts do not return to normal values until Day 21. When r-HuMGDF (*E. coli* 1-163) was administered once daily (100 ug/kg/day) to irradiated mice from Day 1 through Day 20, the drop in platelet counts was less severe and the return to baseline levels more rapid than mice treated with excipient (FIG. 21). In order to test r-HuMGDF in a model of extreme and prolonged thrombocytopenia, carboplatin and irradiation were applied in combination (FIG. 22). In this circumstance, platelet counts dropped to extremely low levels, (3-5% of normal), and most of the animals (7/8) did not survive this treatment. However, when these animals were treated daily with subcutaneous injections of r-HuMGDF at 100 ug/kg/day for the length of the study, thrombocytopenia was significantly abrogated, the return to baseline counts was more rapid, and all of the r-HuMGDF-treated animals (8/8) survived.

r-HuMGDF was also evaluated in rhesus monkeys.

-105-

Normal rhesus monkeys were subcutaneously injected with either 2.5 or 25 ug/kg/day for 10 days (Day 0-9). In the lower dose group, platelet counts increased by 400% at Day 12 and in the higher dose group they increased by 700%, also at Day 12. After the injections stopped, platelet counts returned to normal by Day 25-30. White blood cell counts and red blood cell counts were not affected by this treatment.

r-HuMGDF (*E. coli* 1-163) was also tested in a primate model of severe thrombocytopenia (FIG. 23). Animals were subjected to irradiation (700 rads, Cobalt source) which resulted in a reduction of platelet counts to 1-2% of normal by Day 15. By Day 35-40, platelet counts returned to normal. In contrast, the platelet counts in irradiated animals treated daily with r-HuMGDF (25 ug/kg/day) dropped to only 10% of normal and on average did not go below 20,000/ul, the trigger point for platelet transfusions in thrombocytopenic humans. The return to baseline counts was also more rapid in the r-HuMGDF-treated animals, occurring by Day 20.

These in vivo data from both rodent and primate studies fully support the concept that r-HuMGDF (*E. coli* 1-163) is a potent therapeutic agent with the capacity to significantly affect clinically relevant thrombocytopenias.

EXAMPLE 16

Method for CHO Cell Culture Production of r-HuMGDF 1-332

30

Glycosylated r-Hu MGDF 1-332 is produced from transfected Chinese Hamster Ovary cells expressing a cDNA for MGDF 1-332 under a suitable promoter and linked to a gene coding for the amplifiable selection marker, DHFR. A suitable promoter for expression of MGDF in CHO cells is SR α . See Mol. Cell. Biol. 8: 466-472 (1988)

-106-

and WO 91/13160 (1991). A suitable vector for expression of MGDF in CHO cells is pDSR α 2. See WO 90/14363 (1990). Exemplary CHO cell lines can produce secreted MGDF in the range of 10-20 mg/L in standard cell culture media, but may be increased to 25 to $\geq$ 100 mg/L. To produce MGDF with a typical cell line, a culture can be expanded by passaging in suspension or in tissue culture vessels in adherent growth mode using medium comprised of equal proportions Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (DMEM/F12, Gibco) supplemented with 5 to 10% Fetal Bovine Serum (FBS) or dialyzed fetal bovine serum and methotrexate (MTX) (if necessary; typical concentration of MTX is 200-600 nM) to maintain selection pressure. This media should be supplemented with extra non-essential amino acids (NEAA's) and glutamine. Suspension cultures can propagate readily between inoculation (splitting) densities of $1-4 \times 10^5$ cells/mL and maximal densities of $\sim 1 \times 10^6$ cells/mL at which point the cultures are expanded by dilution into larger volumes with initial cell densities at the specified splitting densities.

To produce MGDF in roller bottles, a suitable volume and cellular density of suspension culture must be generated using either magnetically stirred spinner vessels placed in a temperature controlled environment ($37 \pm 1^\circ\text{C}$), or an instrumented, controlled, stirred-tank bioreactor system. Roller bottles (such as 850 cm² Falcon roller bottles) should be seeded at initial densities of 1.5 to 3×10^7 cells per bottle and supplemented with additional growth medium (DMEM/F12 with 5-10% FBS, 1X NEAA and 1X L-glutamine) in an amount suitable to generate a confluent monolayer in 3-4 days (150-300 mL per bottle). The growth medium should be suitably buffered with sodium bicarbonate to a pH of 6.9 to 7.2 in equilibrium with carbon dioxide at a partial pressure of 60 to 90 mm Hg. Bottles should be gassed

2167090

-107-

with 10% CO₂/air and incubated on roller racks (~1 rpm) at 37 ± 1°C for 3-4 days. At confluence, the roller bottles should be shifted to serum-free production medium by pouring or aspirating the growth medium; washing the bottles with an isotonic buffer such as Dulbecco's Phosphate Buffered Saline (D-PBS), 50-100 mL per bottle; then adding an appropriate volume of bicarbonate-buffered, serum-free DMEM/F12 (1:1) (200 - 300 mL per bottle) supplemented with NEAA's and L-glutamine and with copper sulfate to minimize covalent aggregation (1-20 µM). Bottles should be gassed with 10% CO₂/air and incubated for 6 ± 1 days at 37 ± 1°C on roller racks (~1 rpm), or until metabolic activity has driven the glucose level to below 0.5 g/L and/or the pH level below 6.6. The conditioned medium should be harvested by pouring or aspirating from the bottles and replaced with fresh, serum-free production medium, as described above, for additional harvests. This can proceed until the cells can no longer sustain serum-free production and slough off of the roller bottles.

Harvested conditioned medium can be processed for purification by dead-end microfiltration through 0.45 µm and/or 0.2 µm filters (Sartorius Sartobran pH or Pall). Filtered conditioned medium should be chilled to 4°C, then either stored temporarily at 4°C, or immediately concentrated and dialyzed to low ionic strength using a cross-flow, ultrafiltration system (i.e. Filtron YM-50^{*}). Ultrafiltration and diafiltration should occur at 4°C to minimize protein degradation. Conditioned medium should be dialyzed with a buffered aqueous solution (i.e. 10 mM potassium phosphate, pH 6.8) prior to chromatographic purification steps.

Product quality in conditioned medium can be best monitored using non-reducing SDS-PAGE Western blots which can reveal the relative amounts of aggregated, monomeric, and proteolytically degraded MGDF in the

*Trademark

-108-

samples.

Another method for producing MGDF from CHO cells would be to adapt a cell line expressing MGDF to a serum-free medium such as Gibco S-SFM II. Cells can be adapted by serial passaging in medium containing minimal or no serum supplements. If a cell line is found to grow sustainably in such a medium while producing adequate amounts of secreted MGDF, production can proceed by scaling up an inoculum culture via serial passaging in increasingly larger culture volumes, then inoculating a suitable production vessel (an instrumented, controlled, stirred-tank bioreactor) and allowing the culture to proliferate to its maximal viable density under optimal growth conditions (pH, nutrients, temperature, oxygen, shear). At the optimal production point (as determined experimentally by measuring product quantity and quality) the culture can be harvested from the bioreactor, and the cells can be removed from the conditioned medium by micron-scale depth filtration or sub-micron cross-flow microfiltration. If depth filtration is used the medium should be further clarified by sub-micron dead-end filtration prior to concentration and dialysis as described above.

25

* * *

While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art in light of the above description. Therefore, it is intended that the appended claims cover all such variations coming within the scope of the invention as claimed.

35

-109-

Additionally, the publications and other materials are cited to illuminate the background of the invention, and in particular cases to provide additional details concerning its practice.

5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Bartley, Timothy D.
Bogenberger, Jakob M.
Bosselman, Robert A.
Hunt, Pamela
Kinstler, Olaf B.
Samal, Babru B.
- (ii) TITLE OF INVENTION: Compositions and Methods for Stimulating
Megakaryocyte Growth and Differentiation
- (iii) NUMBER OF SEQUENCES: 34
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Amgen Inc.
 - (B) STREET: 1840 Dehavilland Drive
 - (C) CITY: Thousand Oaks
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 91320-1789
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC*compatible
 - (C) OPERATING SYSTEM: PC-DOS*/MS-DOS*
 - (D) SOFTWARE: PatentIn*Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Cook, Robert R.
 - (C) REFERENCE/DOCKET NUMBER: A-290-C

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Ala	Pro	Pro	Ala	Xaa	Asp	Pro	Arg	Leu	Leu	Asn	Lys	Met	Leu	Arg	Asp
1				5				10					15		

*Trademark

- 111 -

Ser His Val Leu His Xaa Arg Leu Xaa Gln Xaa Pro Asp Ile Tyr
 20 25 30

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Ala Pro Pro Ala Xaa Asp Pro Arg Leu Leu Asn Lys Met Leu Arg Asp
 1 5 10 15

Ser His Val Leu His
 20

(2) INFORMATION FOR SEQ ID NO:3:

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

(ii) MOLECULE TYPE: protein

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

Thr Gln Lys Glu Gln Thr Lys Ala Gln Asp Val Leu Gly Ala Val Ala
 1 5 10 15

Leu

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

GCNCCNCCNG CNTGYGA

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

GCARTGYAAC ACRTGNGART C

21

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Ala	Pro	Pro	Ala	Cys	Asp	Leu	Arg	Val	Leu	Ser	Lys	Leu	Leu	Arg	Asp
1				5					10					15	
Ser	His	Val	Leu	His											
					20										

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTACGCGTTC TAGANNNNNN T

21

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGTTTACTGA GGACTCGGAG G

21

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTCGGCCGGA TAGGCCTTTT TTTTTTTTTT

30

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTCGGCCGGA TAGGCCTTTT TTTTTTTTTT

29

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGCGACCTCC GAGTCCTCAG

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAGTCCTCAG TAAACTGCTT CGT

23

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAGTCACGA AGCAGTTTAC

20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCTTTACTTC TAGGCCTG

18

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAGGTCACAA GCAGGAGGA

19

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGCATAGTCC GGGACGTCG

19

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCCTCCTGCT TGTGACCTC

19

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

2167090

- 116 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCAGGAAGGA TTCAGGGGA

19

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAACAAGTCG ACCGCCAGCC AGACACCCCG

30

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCCGGATAG GCCACTCNNN NNNT

24

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCARTGYAAN ACRTGNGART C

21

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTGGTGTGCA CTTGTG

16

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CACAAGTGCA CACCAACCCC

20

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1342 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 99..1094

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAGGGAGCCA CGCCAGCCAA GACACCCCGG CCAGAATGGA GCTGACTGAA TTGCTCCTCG

60

TGGTCATGCT TCTCCTAACT GCAAGGCTAA CGCTGTCC AGC CCG GCT CCT CCT
 Ser Pro Ala Pro Pro
 1 5

113

2167090

- 118 -

GCT	TGT	GAC	CTC	CGA	GTC	CTC	AGT	AAA	CTG	CTT	CGT	GAC	TCC	CAT	GTC	161
Ala	Cys	Asp	Leu	Arg	Val	Leu	Ser	Lys	Leu	Leu	Arg	Asp	Ser	His	Val	
				10					15					20		
CTT	CAC	AGC	AGA	CTG	AGC	CAG	TGC	CCA	GAG	GTT	CAC	CCT	TTG	CCT	ACA	209
Leu	His	Ser	Arg	Leu	Ser	Gln	Cys	Pro	Glu	Val	His	Pro	Leu	Pro	Thr	
			25					30					35			
CCT	GTC	CTG	CTG	CCT	GCT	GTG	GAC	TTT	AGC	TTG	GGA	GAA	TGG	AAA	ACC	257
Pro	Val	Leu	Leu	Pro	Ala	Val	Asp	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	
		40					45					50				
CAG	ATG	GAG	GAG	ACC	AAG	GCA	CAG	GAC	ATT	CTG	GGA	GCA	GTG	ACC	CTT	305
Gln	Met	Glu	Glu	Thr	Lys	Ala	Gln	Asp	Ile	Leu	Gly	Ala	Val	Thr	Leu	
	55					60					65					
CTG	CTG	GAG	GGA	GTG	ATG	GCA	GCA	CGG	GGA	CAA	CTG	GGA	CCC	ACT	TGC	353
Leu	Leu	Glu	Gly	Val	Met	Ala	Ala	Arg	Gly	Gln	Leu	Gly	Pro	Thr	Cys	
70					75					80					85	
CTC	TCA	TCC	CTC	CTG	GGG	CAG	CTT	TCT	GGA	CAG	GTC	CGT	CTC	CTC	CTT	401
Leu	Ser	Ser	Leu	Leu	Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	Leu	Leu	
				90					95					100		
GGG	GCC	CTG	CAG	AGC	CTC	CTT	GGA	ACC	CAG	CTT	CCT	CCA	CAG	GGC	AGG	449
Gly	Ala	Leu	Gln	Ser	Leu	Leu	Gly	Thr	Gln	Leu	Pro	Pro	Gln	Gly	Arg	
			105					110					115			
ACC	ACA	GCT	CAC	AAG	GAT	CCC	AAT	GCC	ATC	TTC	CTG	AGC	TTC	CAA	CAC	497
Thr	Thr	Ala	His	Lys	Asp	Pro	Asn	Ala	Ile	Phe	Leu	Ser	Phe	Gln	His	
		120					125					130				
CTG	CTC	CGA	GGA	AAG	GTG	CGT	TTC	CTG	ATG	CTT	GTA	GGA	GGG	TCC	ACC	545
Leu	Leu	Arg	Gly	Lys	Val	Arg	Phe	Leu	Met	Leu	Val	Gly	Gly	Ser	Thr	
	135					140				145						
CTC	TGC	GTC	AGG	CGG	GCC	CCA	CCC	ACC	ACA	GCT	GTC	CCC	AGC	AGA	ACC	593
Leu	Cys	Val	Arg	Arg	Ala	Pro	Pro	Thr	Thr	Ala	Val	Pro	Ser	Arg	Thr	
150					155					160					165	
TCT	CTA	GTC	CTC	ACA	CTG	AAC	GAG	CTC	CCA	AAC	AGG	ACT	TCT	GGA	TTG	641
Ser	Leu	Val	Leu	Thr	Leu	Asn	Glu	Leu	Pro	Asn	Arg	Thr	Ser	Gly	Leu	
				170					175					180		
TTG	GAG	ACA	AAC	TTC	ACT	GCC	TCA	GCC	AGA	ACT	ACT	GGC	TCT	GGG	CTT	689
Leu	Glu	Thr	Asn	Phe	Thr	Ala	Ser	Ala	Arg	Thr	Thr	Gly	Ser	Gly	Leu	
			185					190					195			
CTG	AAG	TGG	CAG	CAG	GGA	TTC	AGA	GCC	AAG	ATT	CCT	GGT	CTG	CTG	AAC	737
Leu	Lys	Trp	Gln	Gln	Gly	Phe	Arg	Ala	Lys	Ile	Pro	Gly	Leu	Leu	Asn	
	200					205						210				
CAA	ACC	TCC	AGG	TCC	CTG	GAC	CAA	ATC	CCC	GGA	TAC	CTG	AAC	AGG	ATA	785
Gln	Thr	Ser	Arg	Ser	Leu	Asp	Gln	Ile	Pro	Gly	Tyr	Leu	Asn	Arg	Ile	
	215					220					225					

CAC GAA CTC TTG AAT GGA ACT CGT GGA CTC TTT CCT GGA CCC TCA CGC	833
His Glu Leu Leu Asn Gly Thr Arg Gly Leu Phe Pro Gly Pro Ser Arg	
230 235 240 245	
AGG ACC CTA GGA GCC CCG GAC ATT TCC TCA GGA ACA TCA GAC ACA GGC	881
Arg Thr Leu Gly Ala Pro Asp Ile Ser Ser Gly Thr Ser Asp Thr Gly	
250 255 260	
TCC CTG CCA CCC AAC CTC CAG CCT GGA TAT TCT CCT TCC CCA ACC CAT	929
Ser Leu Pro Pro Asn Leu Gln Pro Gly Tyr Ser Pro Ser Pro Thr His	
265 270 275	
CCT CCT ACT GGA CAG TAT ACG CTC TTC CCT CTT CCA CCC ACC TTG CCC	977
Pro Pro Thr Gly Gln Tyr Thr Leu Phe Pro Leu Pro Pro Thr Leu Pro	
280 285 290	
ACC CCT GTG GTC CAG CTC CAC CCC CTG CTT CCT GAC CCT TCT GCT CCA	1025
Thr Pro Val Val Gln Leu His Pro Leu Leu Pro Asp Pro Ser Ala Pro	
295 300 305	
ACG CCC ACC CCT ACC AGC CCT CTT CTA AAC ACA TCC TAC ACC CAC TCC	1073
Thr Pro Thr Pro Thr Ser Pro Leu Leu Asn Thr Ser Tyr Thr His Ser	
310 315 320 325	
CAG AAT CTG TCT CAG GAA GGG TAAGGTTCTC AGACACTGCC GACATCAGCA	1124
Gln Asn Leu Ser Gln Glu Gly	
330	
TTGTCTCGTG TACAGCTCCC TTCCCTGCAG GGCGCCCCTG GGAGACAACT GGACAAGATT	1184
TCCTACTTTC TCCTGAAACC CAAAGCCCTG GTAAAAGGGA TACACAGGAC TGAAAAGGGA	1244
ATCATTTTTC ACTGTACATT ATAAACCTTC AGAAGCTATT TTTTAAAGCT ATCAGCAATA	1304
CTCATCAGAG CAGCTAGCTC TTTGGTCTAT TTTCTGCA	1342

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 332 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu	
1 5 10 15	
Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val	
20 25 30	
His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu	
35 40 45	

- 120 -

2167090

Gly	Glu	Trp	Lys	Thr	Gln	Met	Glu	Glu	Thr	Lys	Ala	Gln	Asp	Ile	Leu
50						55					60				
Gly	Ala	Val	Thr	Leu	Leu	Leu	Glu	Gly	Val	Met	Ala	Ala	Arg	Gly	Gln
65					70					75					80
Leu	Gly	Pro	Thr	Cys	Leu	Ser	Ser	Leu	Leu	Gly	Gln	Leu	Ser	Gly	Gln
				85					90					95	
Val	Arg	Leu	Leu	Leu	Gly	Ala	Leu	Gln	Ser	Leu	Leu	Gly	Thr	Gln	Leu
			100					105					110		
Pro	Pro	Gln	Gly	Arg	Thr	Thr	Ala	His	Lys	Asp	Pro	Asn	Ala	Ile	Phe
		115					120					125			
Leu	Ser	Phe	Gln	His	Leu	Leu	Arg	Gly	Lys	Val	Arg	Phe	Leu	Met	Leu
	130						135				140				
Val	Gly	Gly	Ser	Thr	Leu	Cys	Val	Arg	Arg	Ala	Pro	Pro	Thr	Thr	Ala
145					150					155					160
Val	Pro	Ser	Arg	Thr	Ser	Leu	Val	Leu	Thr	Leu	Asn	Glu	Leu	Pro	Asn
				165					170					175	
Arg	Thr	Ser	Gly	Leu	Leu	Glu	Thr	Asn	Phe	Thr	Ala	Ser	Ala	Arg	Thr
			180					185					190		
Thr	Gly	Ser	Gly	Leu	Leu	Lys	Trp	Gln	Gln	Gly	Phe	Arg	Ala	Lys	Ile
	195						200					205			
Pro	Gly	Leu	Leu	Asn	Gln	Thr	Ser	Arg	Ser	Leu	Asp	Gln	Ile	Pro	Gly
	210					215					220				
Tyr	Leu	Asn	Arg	Ile	His	Glu	Leu	Leu	Asn	Gly	Thr	Arg	Gly	Leu	Phe
225					230					235					240
Pro	Gly	Pro	Ser	Arg	Arg	Thr	Leu	Gly	Ala	Pro	Asp	Ile	Ser	Ser	Gly
				245					250					255	
Thr	Ser	Asp	Thr	Gly	Ser	Leu	Pro	Pro	Asn	Leu	Gln	Pro	Gly	Tyr	Ser
			260					265					270		
Pro	Ser	Pro	Thr	His	Pro	Pro	Thr	Gly	Gln	Tyr	Thr	Leu	Phe	Pro	Leu
		275					280					285			
Pro	Pro	Thr	Leu	Pro	Thr	Pro	Val	Val	Gln	Leu	His	Pro	Leu	Leu	Pro
	290					295					300				
Asp	Pro	Ser	Ala	Pro	Thr	Pro	Thr	Pro	Thr	Ser	Pro	Leu	Leu	Asn	Thr
305					310					315					320
Ser	Tyr	Thr	His	Ser	Gln	Asn	Leu	Ser	Gln	Glu	Gly				
				325					330						

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1342 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 99..621

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```

CAGGGAGCCA CGCCAGCCAA GACACCCCGG CCAGAATGGA GCTGACTGAA TTGCTCCTCG      60
TGGTCATGCT TCTCCTAACT GCAAGGCTAA CGCTGTCC AGC CCG GCT CCT CCT      113
                               Ser Pro Ala Pro Pro
                               1           5

GCT TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC      161
Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val
                        10                15                20

CTT CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA      209
Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr
                        25                30                35

CCT GTC CTG CTG CCT GCT GTG GAC TTT AGC TTG GGA GAA TGG AAA ACC      257
Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr
                        40                45                50

CAG ATG GAG GAG ACC AAG GCA CAG GAC ATT CTG GGA GCA GTG ACC CTT      305
Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu Gly Ala Val Thr Leu
                        55                60                65

CTG CTG GAG GGA GTG ATG GCA GCA CGG GGA CAA CTG GGA CCC ACT TGC      353
Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Gly Pro Thr Cys
                        70                75                80                85

CTC TCA TCC CTC CTG GGG CAG CTT TCT GGA CAG GTC CGT CTC CTC CTT      401
Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu
                        90                95                100

GGG GCC CTG CAG AGC CTC CTT GGA ACC CAG CTT CCT CCA CAG GGC AGG      449
Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu Pro Pro Gln Gly Arg
                        105                110                115

ACC ACA GCT CAC AAG GAT CCC AAT GCC ATC TTC CTG AGC TTC CAA CAC      497
Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe Leu Ser Phe Gln His
                        120                125                130

```

2167090

- 122 -

TG CTC CGA GGA AAG GTG CGT TTC CTG ATG CTT GTA GGA GGG TCC ACC	545
Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu Val Gly Gly Ser Thr	
135 140 145	
CTC TGC GTC AGG CGG GCC CCA CCC ACC ACA GCT GTC CCC AGC AGA ACC	593
Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala Val Pro Ser Arg Thr	
150 155 160 165	
TCT CTA GTC CTC ACA CTG AAC GAG CTC C CAAACAGGAC TTCTGGATTG	641
Ser Leu Val Leu Thr Leu Asn Glu Leu	
170	
TTGGAGACAA ACTTCACTGC CTCAGCCAGA ACTACTGGCT CTGGGCTTCT GAAGTGGCAG	701
CAGGGATTCA GAGCCAAGAT TCCTGGTCTG CTGAACCAAA CCTCCAGGTC CCTGGACCAA	761
ATCCCCGGAT ACCTGAACAG GATACACGAA CTCTTGAATG GAACTCGTGG ACTCTTTCCT	821
GGACCCTCAC GCAGGACCCT AGGAGCCCCG GACATTTTCCT CAGGAACATC AGACACAGGC	881
TCCCTGCCAC CCAACCTCCA GCCTGGATAT TCTCCTTCCC CAACCCATCC TCCTACTGGA	941
CAGTATACGC TCTTCCCTCT TCCACCCACC TTGCCCACCC CTGTGGTCCA GCTCCACCCC	1001
CTGCTTCCTG ACCCTTCTGC TCCAACGCCC ACCCCTACCA GCCCTCTTCT AAACACATCC	1061
TACACCCACT CCCAGAATCT GTCTCAGGAA GGGTAAGGTT CTCAGACACT GCCGACATCA	1121
GCATTGTCTC GTGTACAGCT CCCTTCCCTG CAGGGCGCCC CTGGGAGACA ACTGGACAAG	1181
ATTCCTACT TTCTCCTGAA ACCCAAAGCC CTGGTAAAAG GGATACACAG GACTGAAAAG	1241
GGAATCATTT TTTACTGTAC ATTATAAACC TTCAGAAGCT ATTTTTTTTAA GCTATCAGCA	1301
ATACTCATCA GAGCAGCTAG CTCTTTGGTC TATTTTCTGC A	1342

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 174 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu
1 5 10 15
Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val
20 25 30
His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu
35 40 45

- 123 -

Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu
 50 55 60
 Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln
 65 70 75 80
 Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln
 85 90 95
 Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu
 100 105 110
 Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe
 115 120 125
 Leu Ser Phe Gln His Leu Leu Arg Gly Lys Val Arg Phe Leu Met Leu
 130 135 140
 Val Gly Gly Ser Thr Leu Cys Val Arg Arg Ala Pro Pro Thr Thr Ala
 145 150 155 160
 Val Pro Ser Arg Thr Ser Leu Val Leu Thr Leu Asn Glu Leu
 165 170

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1164 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 97..894

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGGGAGCCAC GCCAGCCAGA CACCCCGGCC AGAATGGAGC TGACTGAATT GTCCTCGTG 60
 GTCATGCTTC TCCTAACTGC AAGGCTAACG CTGTCC AGC CCG GCT CCT CCT GCT 114
 Ser Pro Ala Pro Pro Ala
 1 5
 TGT GAC CTC CGA GTC CTC AGT AAA CTG CTT CGT GAC TCC CAT GTC CTT 162
 Cys Asp Leu Arg Val Leu Ser Lys Leu Leu Arg Asp Ser His Val Leu
 10 15 20
 CAC AGC AGA CTG AGC CAG TGC CCA GAG GTT CAC CCT TTG CCT ACA CCT 210
 His Ser Arg Leu Ser Gln Cys Pro Glu Val His Pro Leu Pro Thr Pro
 25 30 35

2167090

- 124 -

GTC	CTG	CTG	CCT	GCT	GTG	GAC	TTT	AGC	TTG	GGA	GAA	TGG	AAA	ACC	CAG	258
Val	Leu	Leu	Pro	Ala	Val	Asp	Phe	Ser	Leu	Gly	Glu	Trp	Lys	Thr	Gln	
	40					45					50					
ATG	GAG	GAG	ACC	AAG	GCA	CAG	GAC	ATT	CTG	GGA	GCA	GTG	ACC	CTT	CTG	306
Met	Glu	Glu	Thr	Lys	Ala	Gln	Asp	Ile	Leu	Gly	Ala	Val	Thr	Leu	Leu	
55					60					65					70	
CTG	GAG	GGA	GTG	ATG	GCA	GCA	CGG	GGA	CAA	CTG	GGA	CCC	ACT	TGC	CTC	354
Leu	Glu	Gly	Val	Met	Ala	Ala	Arg	Gly	Gln	Leu	Gly	Pro	Thr	Cys	Leu	
				75					80					85		
TCA	TCC	CTC	CTG	GGG	CAG	CTT	TCT	GGA	CAG	GTC	CGT	CTC	CTC	CTT	GGG	402
Ser	Ser	Leu	Leu	Gly	Gln	Leu	Ser	Gly	Gln	Val	Arg	Leu	Leu	Leu	Gly	
			90					95					100			
GCC	CTG	CAG	AGC	CTC	CTT	GGA	ACC	CAG	CTT	CCT	CCA	CAG	GGC	AGG	ACC	450
Ala	Leu	Gln	Ser	Leu	Leu	Gly	Thr	Gln	Leu	Pro	Pro	Gln	Gly	Arg	Thr	
		105					110					115				
ACA	GCT	CAC	AAG	GAT	CCC	AAT	GCC	ATC	TTC	CTG	AGC	TTC	CAA	CAC	CTG	498
Thr	Ala	His	Lys	Asp	Pro	Asn	Ala	Ile	Phe	Leu	Ser	Phe	Gln	His	Leu	
	120					125					130					
CTC	CGA	GGA	AAG	GAC	TTC	TGG	ATT	GTT	GGA	GAC	AAA	CTT	CAC	TGC	CTC	546
Leu	Arg	Gly	Lys	Asp	Phe	Trp	Ile	Val	Gly	Asp	Lys	Leu	His	Cys	Leu	
135					140					145					150	
AGC	CAG	AAC	TAC	TGG	CTC	TGG	GCT	TCT	GAA	GTG	GCA	GCA	GGG	ATT	CAG	594
Ser	Gln	Asn	Tyr	Trp	Leu	Trp	Ala	Ser	Glu	Val	Ala	Ala	Gly	Ile	Gln	
				155					160					165		
AGC	CAA	GAT	TCC	TGG	TCT	GCT	GAA	CCA	AAC	CTC	CAG	GTC	CCT	GGA	CCA	642
Ser	Gln	Asp	Ser	Trp	Ser	Ala	Glu	Pro	Asn	Leu	Gln	Val	Pro	Gly	Pro	
			170					175					180			
AAT	CCC	CGG	ATA	CCT	GAA	CAG	GAT	ACA	CGA	ACT	CTT	GAA	TGG	AAC	TCG	690
Asn	Pro	Arg	Ile	Pro	Glu	Gln	Asp	Thr	Arg	Thr	Leu	Glu	Trp	Asn	Ser	
		185					190					195				
TGG	ACT	CTT	TCC	TGG	ACC	CTC	ACG	CAG	GAC	CCT	AGG	AGC	CCC	GGA	CAT	738
Trp	Thr	Leu	Ser	Trp	Thr	Leu	Thr	Gln	Asp	Pro	Arg	Ser	Pro	Gly	His	
	200					205					210					
TTC	CTC	AGG	AAC	ATC	AGA	CAC	AGG	CTC	CCT	GCC	ACC	CAA	CCT	CCA	GCC	786
Phe	Leu	Arg	Asn	Ile	Arg	His	Arg	Leu	Pro	Ala	Thr	Gln	Pro	Pro	Ala	
215					220					225					230	
TGG	ATA	TTC	TCC	TTC	CCC	AAC	CCA	TCC	TCC	TAC	TGG	ACA	GTA	TAC	GCT	834
Trp	Ile	Phe	Ser	Phe	Pro	Asn	Pro	Ser	Ser	Tyr	Trp	Thr	Val	Tyr	Ala	
				235				240						245		
CTT	CCC	TCT	TCC	ACC	CAC	CTT	GCC	CAC	CCC	TGT	GGT	CCA	GCT	CCA	CCC	882
Leu	Pro	Ser	Ser	Thr	His	Leu	Ala	His	Pro	Cys	Gly	Pro	Ala	Pro	Pro	
			250					255					260			

CCT GCT TCC TGACCCTTCT GCTCCAACGC CCACCCCTAC CAGCCCTCTT 931
 Pro Ala Ser
 265

CTAAACACAT CCTACACCCA CTCCCAGAAT CTGTCTCAGG AAGGGTAAGG TTCTCAGACA 991
 CTGCCGACAT CAGCATTGTC TCGTGTACAG CTCCCTTCCC TGCAGGGCGC CCCTGGGAGA 1051
 CAACTGGACA AGATTTCTTA CTTTCTCCTG AAACCCAAAG CCCTGGTAAA AGGGATACAC 1111
 AGGACTGAAA AGGGAATCAT TTTTCACTGT ACATTATAAA CCTTCAGAAG CTA 1164

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 265 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Ser Pro Ala Pro Pro Ala Cys Asp Leu Arg Val Leu Ser Lys Leu Leu
 1 5 10 15

Arg Asp Ser His Val Leu His Ser Arg Leu Ser Gln Cys Pro Glu Val
 20 25 30

His Pro Leu Pro Thr Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu
 35 40 45

Gly Glu Trp Lys Thr Gln Met Glu Glu Thr Lys Ala Gln Asp Ile Leu
 50 55 60

Gly Ala Val Thr Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln
 65 70 75 80

Leu Gly Pro Thr Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln
 85 90 95

Val Arg Leu Leu Leu Gly Ala Leu Gln Ser Leu Leu Gly Thr Gln Leu
 100 105 110

Pro Pro Gln Gly Arg Thr Thr Ala His Lys Asp Pro Asn Ala Ile Phe
 115 120 125

Leu Ser Phe Gln His Leu Leu Arg Gly Lys Asp Phe Trp Ile Val Gly
 130 135 140

Asp Lys Leu His Cys Leu Ser Gln Asn Tyr Trp Leu Trp Ala Ser Glu
 145 150 155 160

Val Ala Ala Gly Ile Gln Ser Gln Asp Ser Trp Ser Ala Glu Pro Asn
 165 170 175

2167090

- 126 -

Leu Gln Val Pro Gly Pro Asn Pro Arg Ile Pro Glu Gln Asp Thr Arg
 180 185 190
 Thr Leu Glu Trp Asn Ser Trp Thr Leu Ser Trp Thr Leu Thr Gln Asp
 195 200 205
 Pro Arg Ser Pro Gly His Phe Leu Arg Asn Ile Arg His Arg Leu Pro
 210 215 220
 Ala Thr Gln Pro Pro Ala Trp Ile Phe Ser Phe Pro Asn Pro Ser Ser
 225 230 235 240
 Tyr Trp Thr Val Tyr Ala Leu Pro Ser Ser Thr His Leu Ala His Pro
 245 250 255
 Cys Gly Pro Ala Pro Pro Pro Ala Ser
 260 265

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GAGCTCACTA GTGTCGACCT GCAG

24

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTGCAGGTCG AACTAGTGA GCTC

24

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 80 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTCATAATTT TTAAAAAATT CATTTGACAA ATGCTAAAAT TCTTGATTAA TATTCTCAAT 60
TGTGAGCGCT CACAATTTAT 80

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 86 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CGATAAATTG TGAGCGCTCA CAATTGAGAA TATTAAATCAA GAATTTTAGC ATTTGTCAAA 60
TGAATTTTTT AAAAATTATG AGACGT 86

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GACGTCTCAT AATTTTAAA AAATTCATTT GACAAATGCT AAAATTCTTG ATTAATATTC 60
TCAATTGTGA GCGCTCACAA TTTATCGAT 89

2 1 6 7 0 9 0

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

1. An MGDF polypeptide, which has an amino acid sequence of a member selected from the group consisting of MGDF-2, MGDF-4, MGDF-5, MGDF-6, MGDF-7, AND MGDF-8.
2. An isolated polynucleotide, which encodes an MGDF polypeptide according to Claim 1.
3. An isolated polynucleotide according to Claim 2, which is a DNA sequence.
4. A DNA sequence according to Claim 3, which is a cDNA sequence.
5. A cDNA sequence according to Claim 4, which has a corresponding sequence as shown in FIG 11.
6. A DNA vector comprising a DNA sequence according to Claim 4.
7. The vector of Claim 6 wherein said DNA sequence is operatively linked to an expression control DNA sequence.
8. A host cell stably transformed or transfected with a DNA sequence according to Claim 4.
9. A host cell according to Claim 8, which expresses said MGDF polypeptide encoded by said DNA sequence.
10. A method for producing an MGDF polypeptide, said method comprising growing a host cell according to Claim 9 in a suitable nutrient medium and isolating said MGDF polypeptide from said cell or said nutrient medium.
11. A method for producing an MGDF polypeptide according to Claim 10, wherein said host cell is an *E. coli* cell.
12. A method for producing an MGDF polypeptide according to Claim 10, wherein said host cell is a CHO cell.

- 2 -

13. An antibody reactive with an MGDF polypeptide according to Claim 1.

14. An MGDF derivative comprising an MGDF polypeptide connected to at least one water soluble polymer, wherein said water soluble polymer is selected from the group consisting of dextran, poly(N-vinyl-pyrrolidone), polyethylene glycols, polypropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols, polyvinyl alcohols and mixtures thereof; and wherein said MGDF polypeptide is a polypeptide within the amino acid sequence defined in Figs. 11 or 12.

15. An MGDF derivative of Claim 14, wherein said MGDF polypeptide is selected from the group consisting of MGDF-1, MGDF-2, MGDF-4, MGDF-11, MGDF-12, MGDF-13, MGDF-14, and MGDF-15.

16. An MGDF derivative of Claim 14, wherein said MGDF polypeptide is recombinantly produced in a bacterial cell.

17. An MGDF derivative of Claim 14, wherein said water soluble polymer is pharmaceutically acceptable.

18. An MGDF derivative of Claim 14, wherein said water soluble polymer is a polyethylene glycol.

19. An MGDF derivative according to Claim 18, wherein said polyethylene glycol is a monomethoxy-polyethylene glycol.

20. An MGDF derivative according to Claim 18, wherein said polyethylene glycol is attached to said MGDF polypeptide by an acyl or an alkyl linkage.

21. An MGDF derivative according to Claim 20, wherein the polyethylene glycol group is attached to the N-terminus thereof.

22. An MGDF derivative according to Claim 20, wherein the polyethylene glycol group has an average molecular weight of 10 to 50 kilodaltons.

23. An MGDF derivative according to Claim 14, comprising an MGDF polypeptide covalently attached to two water soluble polymer molecules.

24. An MGDF derivative according to Claim 23, wherein said water soluble polymer molecules are both polyethylene glycols.

25. A method for attaching a water soluble polymer to an MGDF polypeptide to produce an MGDF derivative according to Claim 14, wherein said water soluble polymer has a reactive aldehyde group, said method comprising reacting a terminal aldehyde derivative of polyethylene glycol with an MGDF polypeptide in the presence of a reducing agent and isolating said MGDF polypeptide attached to at least one water soluble polymer.

26. A method for attaching a water soluble polymer to an MGDF polypeptide to produce an MGDF derivative according to Claim 14, wherein said water soluble polymer has a single reactive aldehyde group, said method comprising:

(a) contacting said MGDF polypeptide with a water soluble polymer under reductive alkylation conditions, at a pH sufficiently acidic to allow the α -amino group at the amino terminus of said MGDF polypeptide to be reactive; and

(b) isolating said MGDF polypeptide attached to at least one water soluble polymer.

27. A method for attaching a water soluble polymer to an MGDF polypeptide to produce an MGDF derivative according to Claim 14, wherein said water soluble polymer has a single reactive ester group, said method comprising:

(a) contacting said MGDF polypeptide with a water soluble polymer under conditions so that said MGDF polypeptide becomes attached to the water soluble polymer through an acyl linkage; and

(b) isolating an MGDF polypeptide attached to at least one water soluble polymer.

28. A method of any of Claims 25 through 27, wherein said polymer is pharmaceutically acceptable.

29. A method of any of Claims 25 through 27, wherein said water soluble polymer is selected from the group consisting of dextran, poly(N-vinyl-pyrrolidone), polyethylene glycols, polypropylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols and polyvinyl alcohols.

30. A method of any of Claims 25 through 27, wherein said water soluble polymer is polyethylene glycol.

31. Mono-pegylated MGDF polypeptide wherein said MGDF polypeptide is a polypeptide within the amino acid sequence defined in Fig. 11 or 12.

32. A mono-pegylated MGDF polypeptide of Claim 31, wherein said MGDF polypeptide is selected from the group consisting of MGDF-1, MGDF-2, MGDF-3, MGDF-4, MGDF-5, MGDF-6, MGDF-7, MGDF-8, MGDF-11, MGDF-12, MGDF-13, MGDF-14, and MGDF-15.

33. A method for attaching a polyethylene glycol molecule to an MGDF polypeptide to produce a mono-pegylated MGDF polypeptide according to Claim 31, wherein said polyethylene glycol molecule has a single reactive aldehyde group, said method comprising:

- (a) contacting said MGDF polypeptide with said polyethylene glycol molecule under reductive alkylation conditions, at a pH sufficiently acidic to allow the α -amino group at the amino terminus of said MGDF polypeptide to be reactive; and
- (b) obtaining a pegylated MGDF polypeptide.

34. A method according to Claim 33, wherein the MGDF polypeptide is made by cleaving Met⁻²-Lys⁻¹ from a polypeptide obtained by expression in an *E. coli* cell of a DNA encoding a polypeptide comprising amino acids 22-184 of Figure 11 and the sequence of Met-Lys at the N-terminus thereof.

35. A method according to Claim 33, wherein the MGDF polypeptide is made by:

- 5 -

a. expressing in an *E. coli* cell a DNA encoding a polypeptide comprising amino acids 22-184 of Figure 11 and the sequence of Met-Lys at the N-terminus thereof,

b. isolating the expressed polypeptide, and

c. cleaving Met⁻²-Lys⁻¹ from the isolated polypeptide.

36. A method of Claim 30 or 33, wherein said polyethylene glycol molecule has a molecular weight of 2 to 100 kilodaltons.

37. A mono-pegylated MGDF polypeptide product produced by the method of Claim 33.

38. A substantially homogeneous preparation of an MGDF polypeptide according to Claim 31 mono-pegylated at the α -amino group at the N-terminus of said MGDF polypeptide.

39. A preparation of Claim 38, wherein said MGDF polypeptide is mono-pegylated with a polyethylene glycol having an average molecular weight of 5 to 50 kilodaltons.

40. A mono-pegylated MGDF polypeptide according to Claim 31, wherein the polyethylene glycol group is attached to the N-terminus of the polypeptide.

41. A mono-pegylated MGDF polypeptide according to Claim 31, wherein the MGDF polypeptide has been produced in *E. coli*.

42. A pharmaceutical composition comprising an MGDF derivative according to Claim 14 and a pharmaceutically acceptable diluent, adjuvant or carrier.

43. A pharmaceutical composition comprising a mono-pegylated MGDF polypeptide according to Claim 31 and a pharmaceutically acceptable diluent, adjuvant or carrier.

44. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for treating a deficiency of MGDF polypeptide in a patient in need thereof.

45. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for the production of a medicament, for treating a deficiency of MGDF polypeptide in a patient in need thereof.

46. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for treating a thrombocytopenic condition in a patient in need thereof.

47. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for the production of a medicament, for treating a thrombocytopenic condition in a patient in need thereof.

48. A use according to Claim 46, wherein said condition is selected from the group consisting of aplastic anemia, idiopathic thrombocytopenia, and thrombocytopenia resulting from drug or radiation treatment.

49. A use according to Claim 47, wherein said condition is selected from the group consisting of aplastic anemia, idiopathic thrombocytopenia, and thrombocytopenia resulting from drug or radiation treatment.

50. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for increasing the number of mature megakaryocytes in a patient in need thereof.

51. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for the

production of a medicament, for increasing the number of mature megakaryocytes in a patient in need thereof.

52. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for increasing the number of platelets in a patient in need thereof.

53. A use of an effective amount of an MGDF polypeptide according to Claim 1, or an MGDF derivative according to Claim 14, for the production of a medicament, for increasing the number of platelets in a patient in need thereof.

1 / 2 8

FIG. 1

Megakaryocyte and Platelet Development

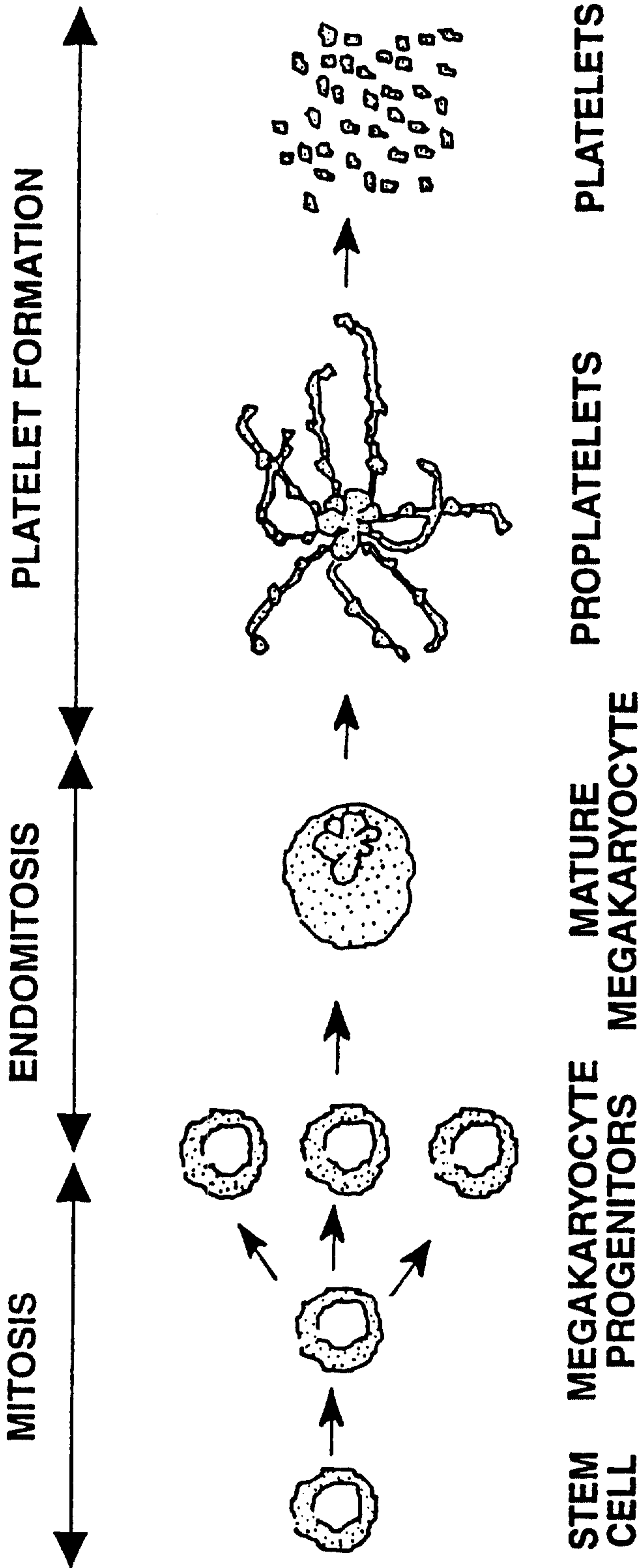
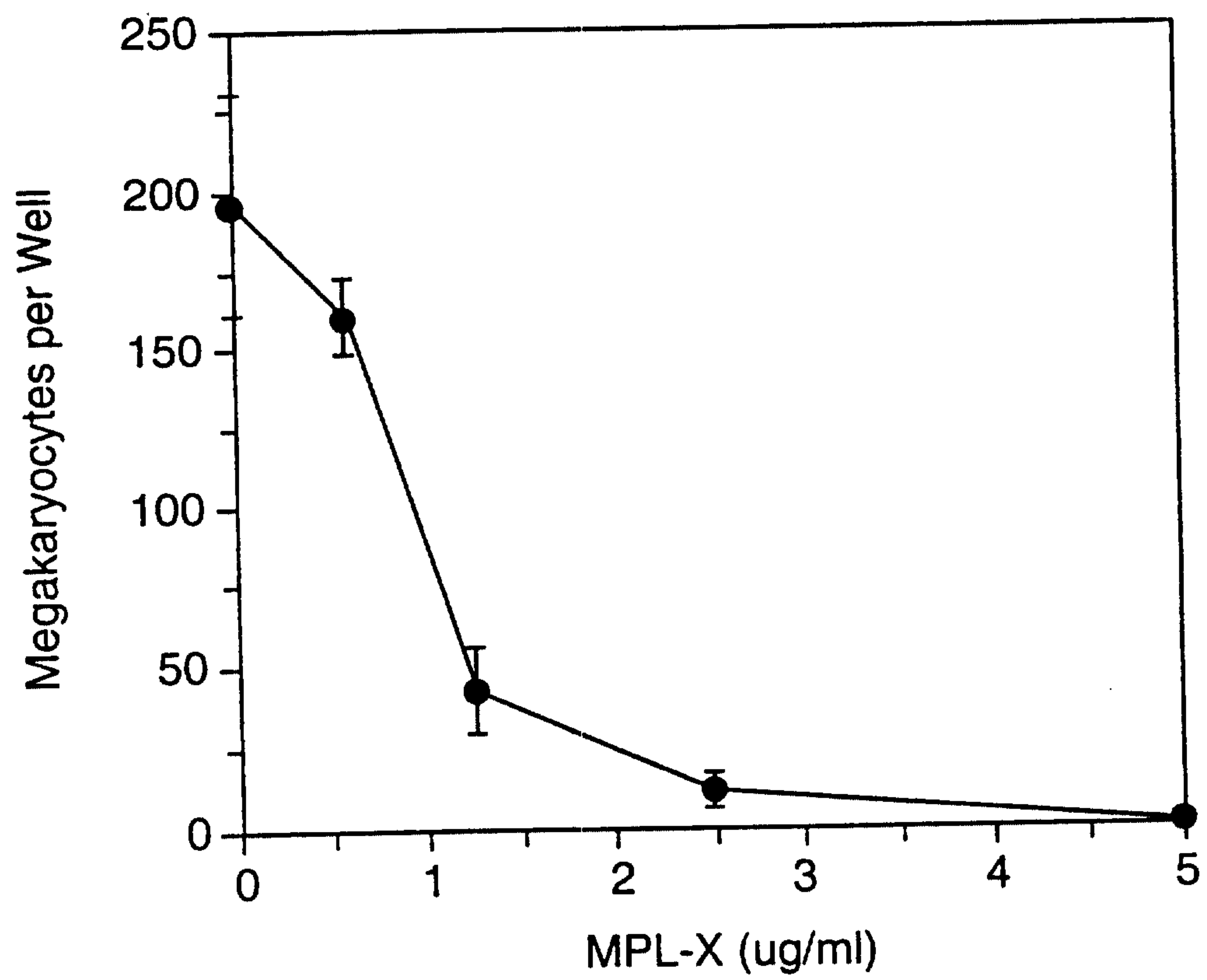


FIG. 2



3 / 2 8

FIG. 3

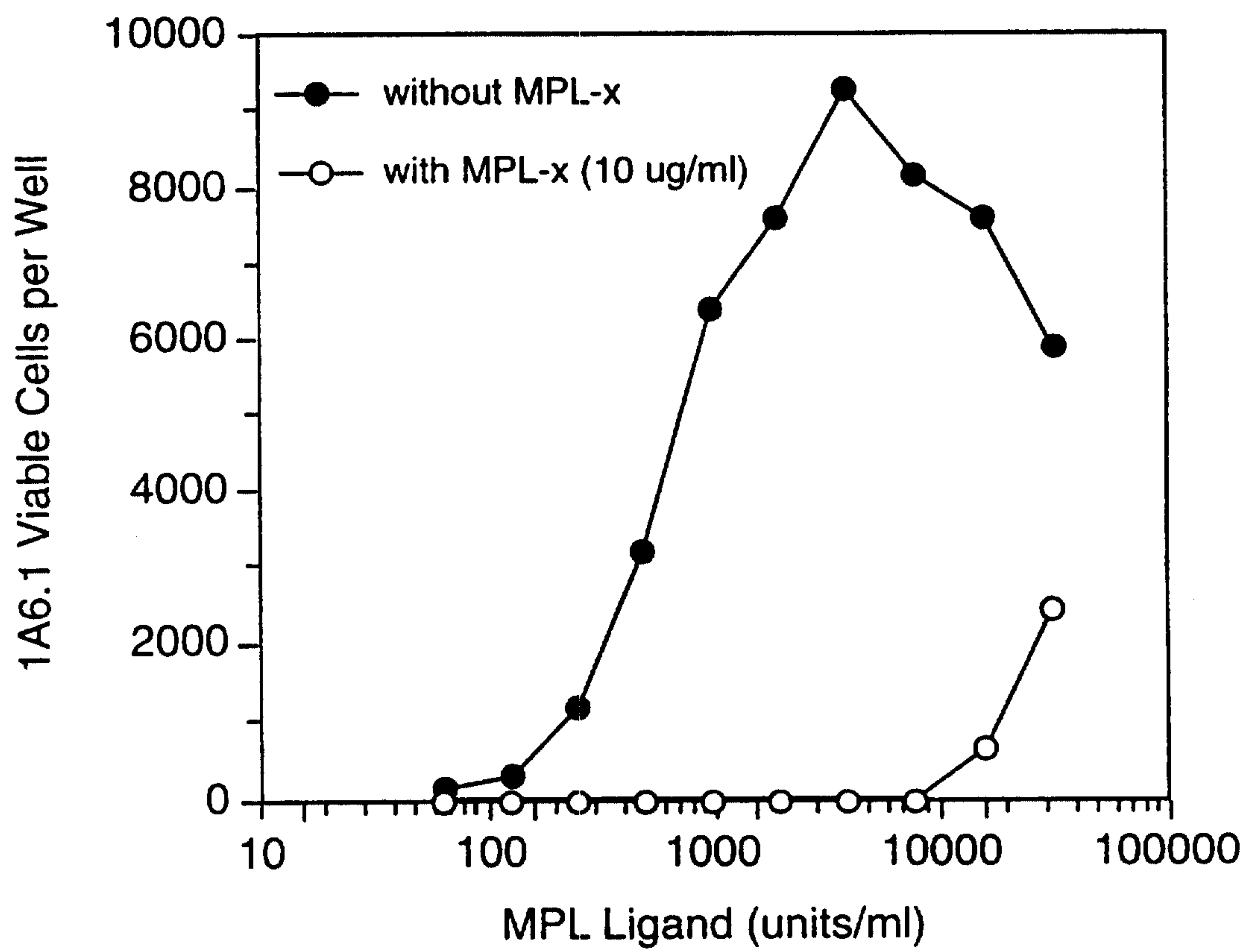


FIG. 4

Purification of Mpl Ligand

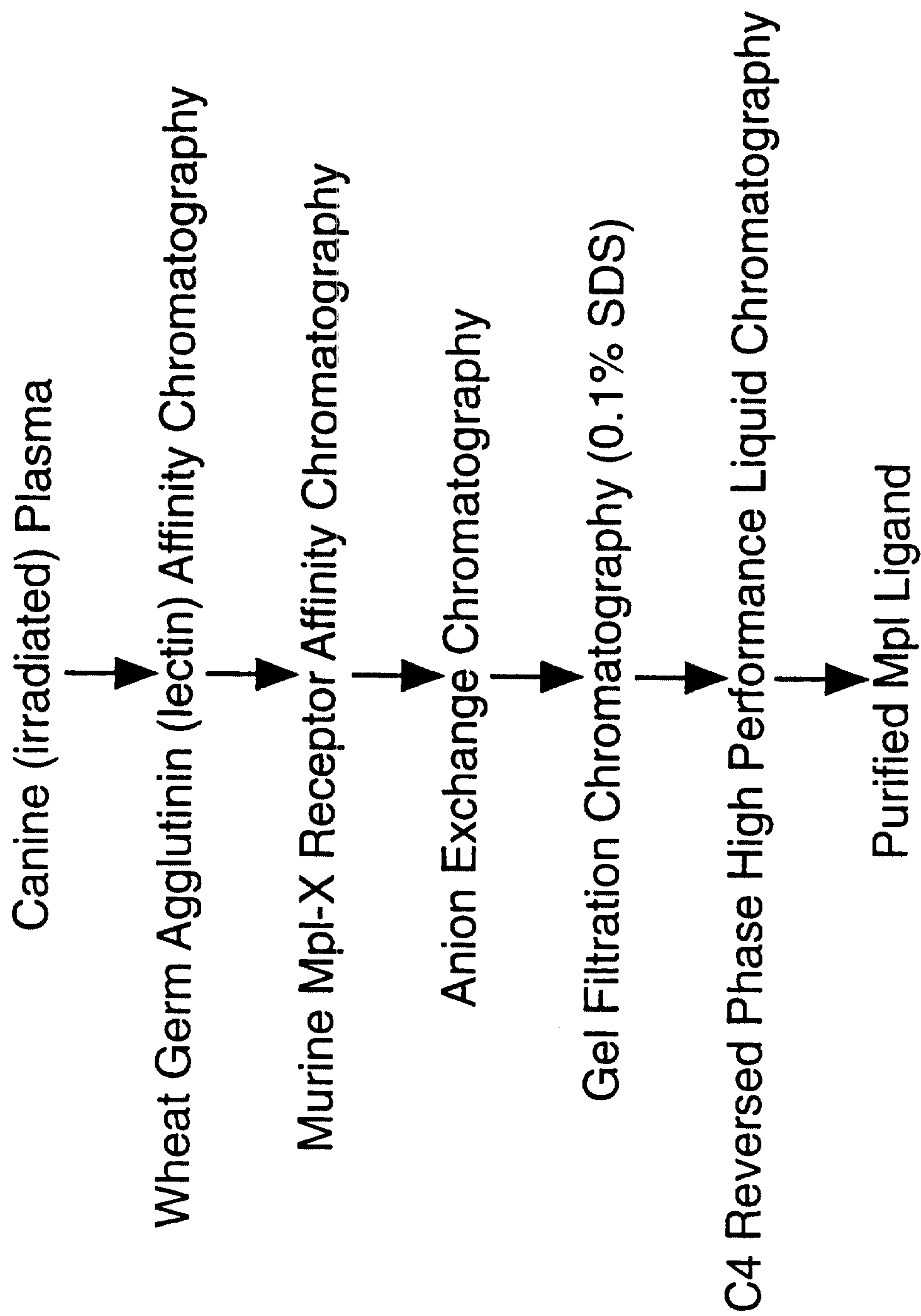


FIG. 5A

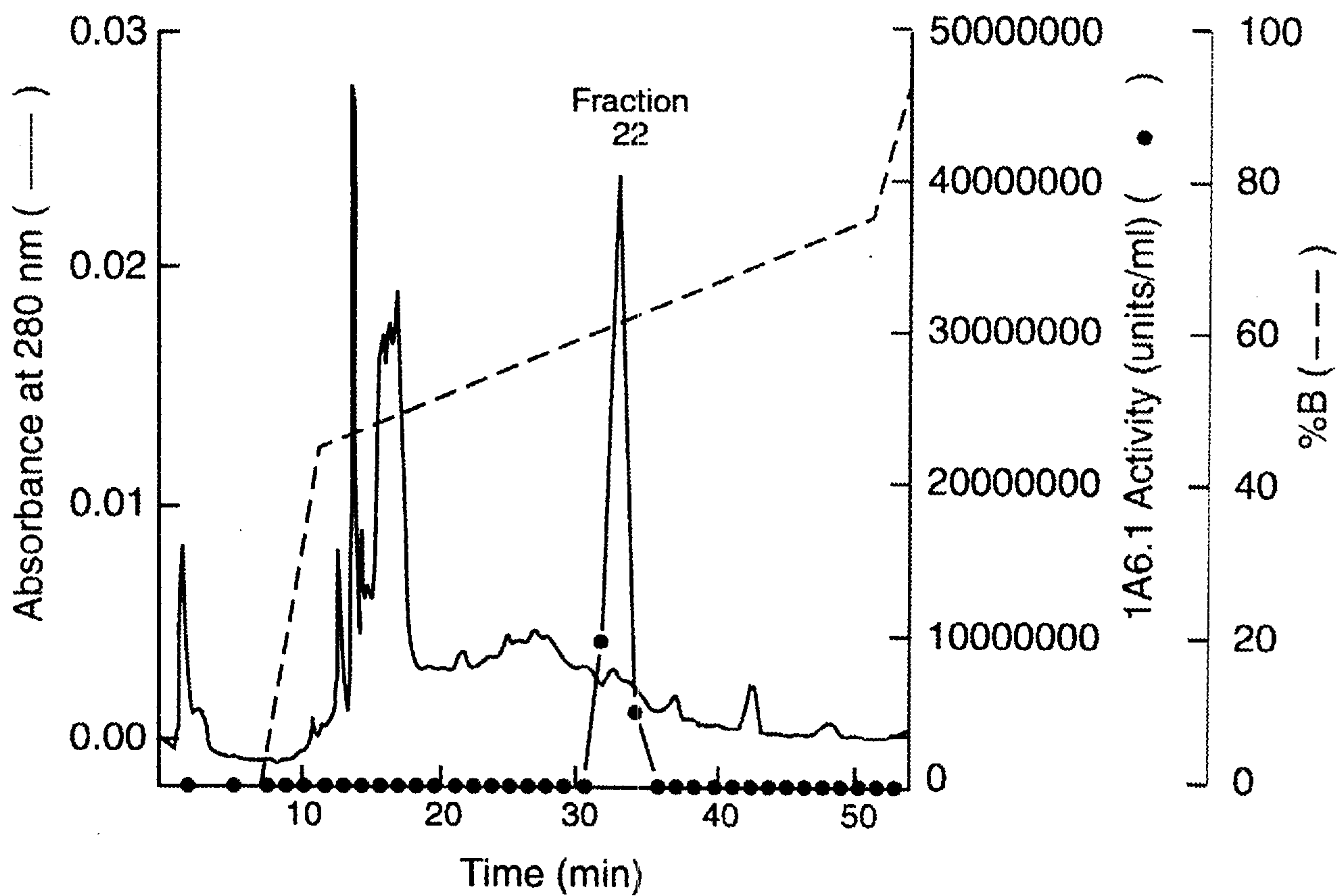


FIG. 5B

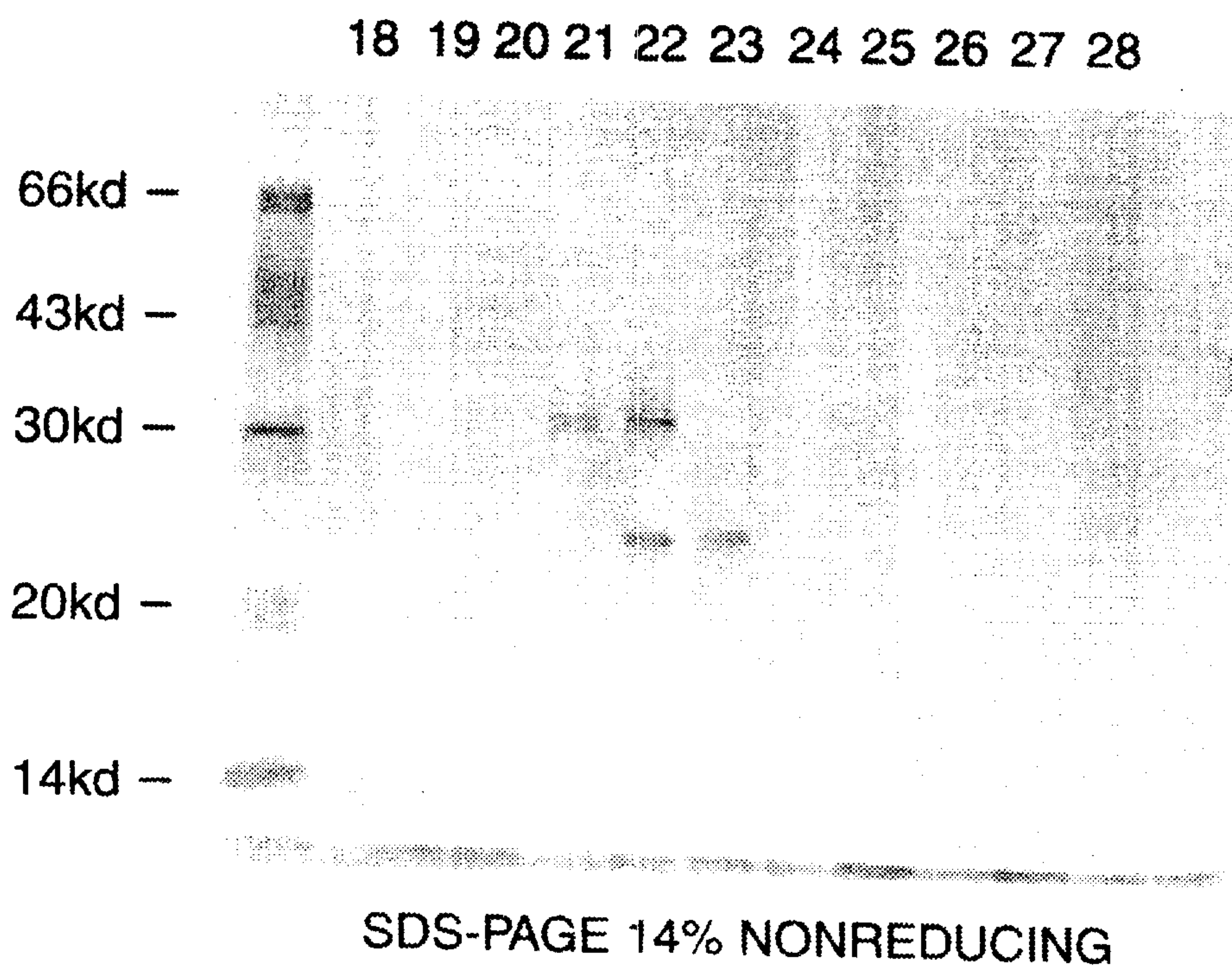


FIG. 6

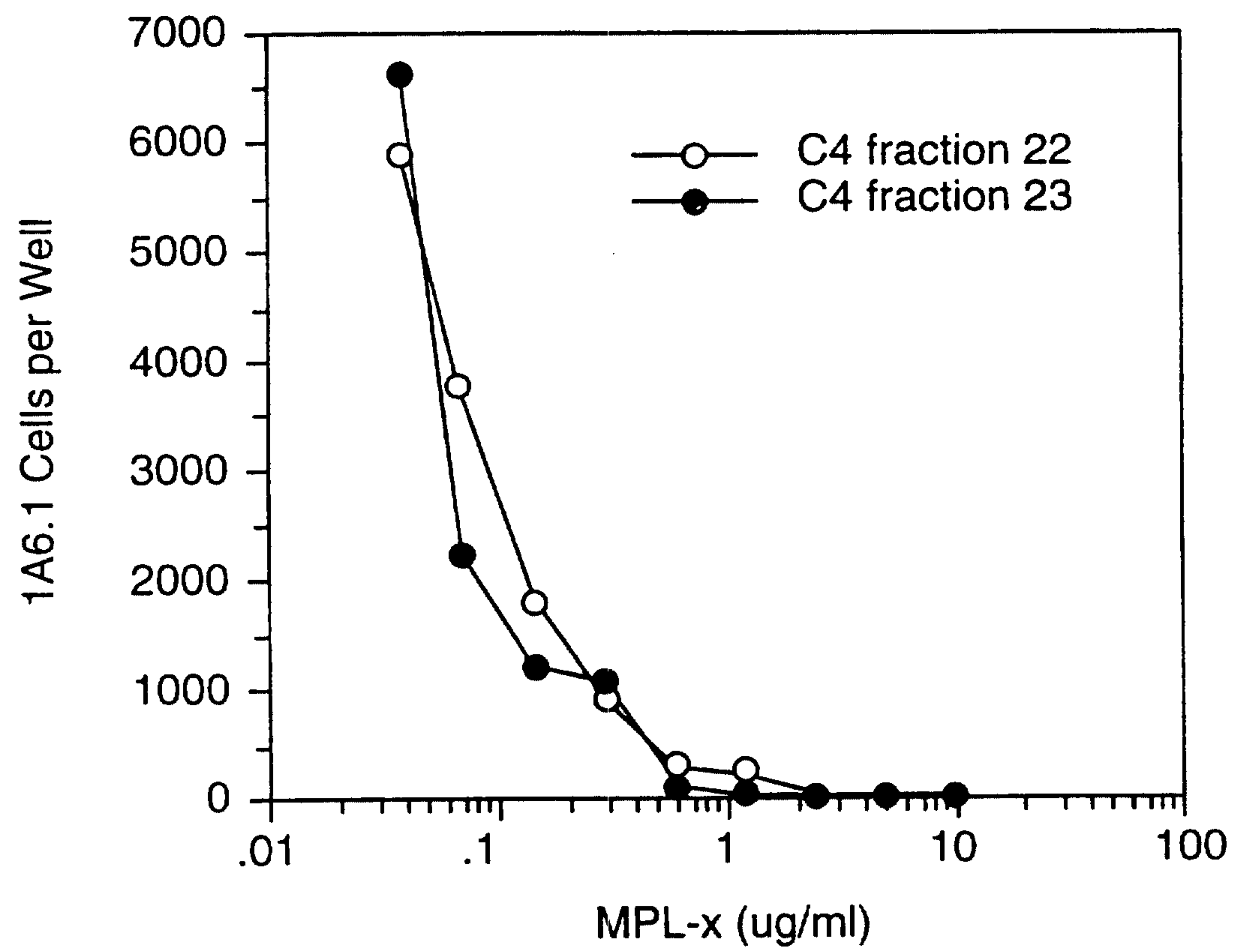


FIG. 7

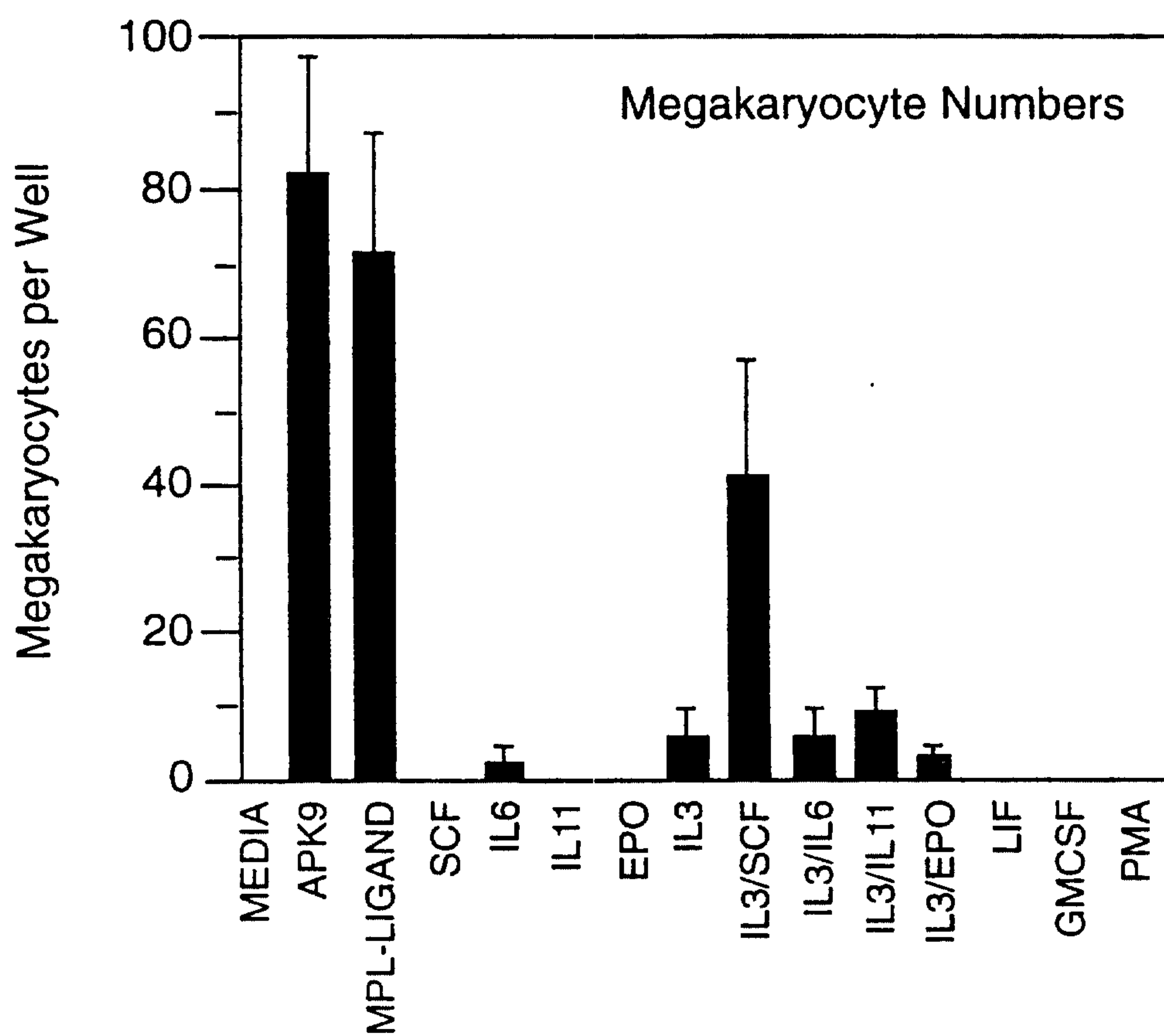
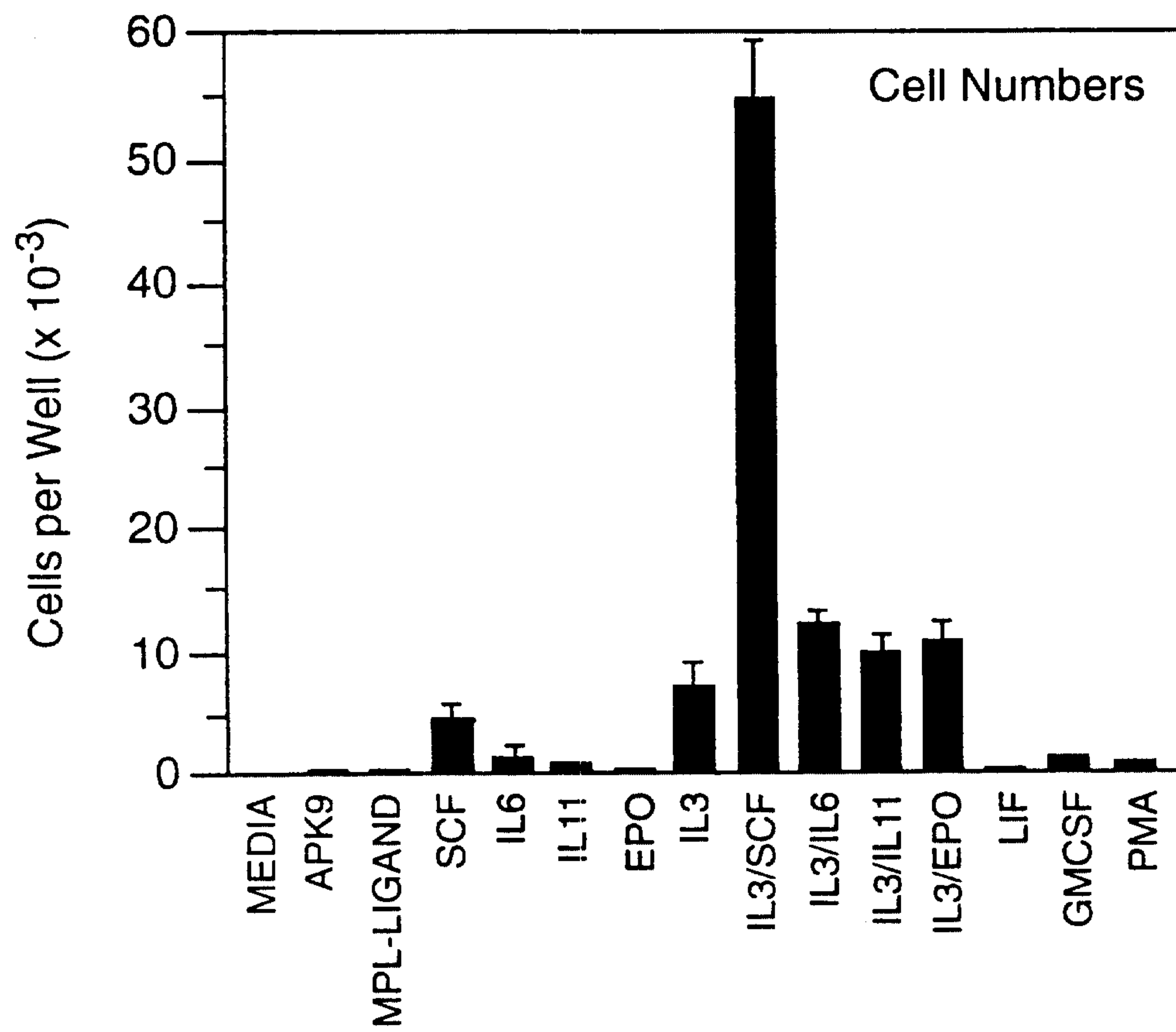
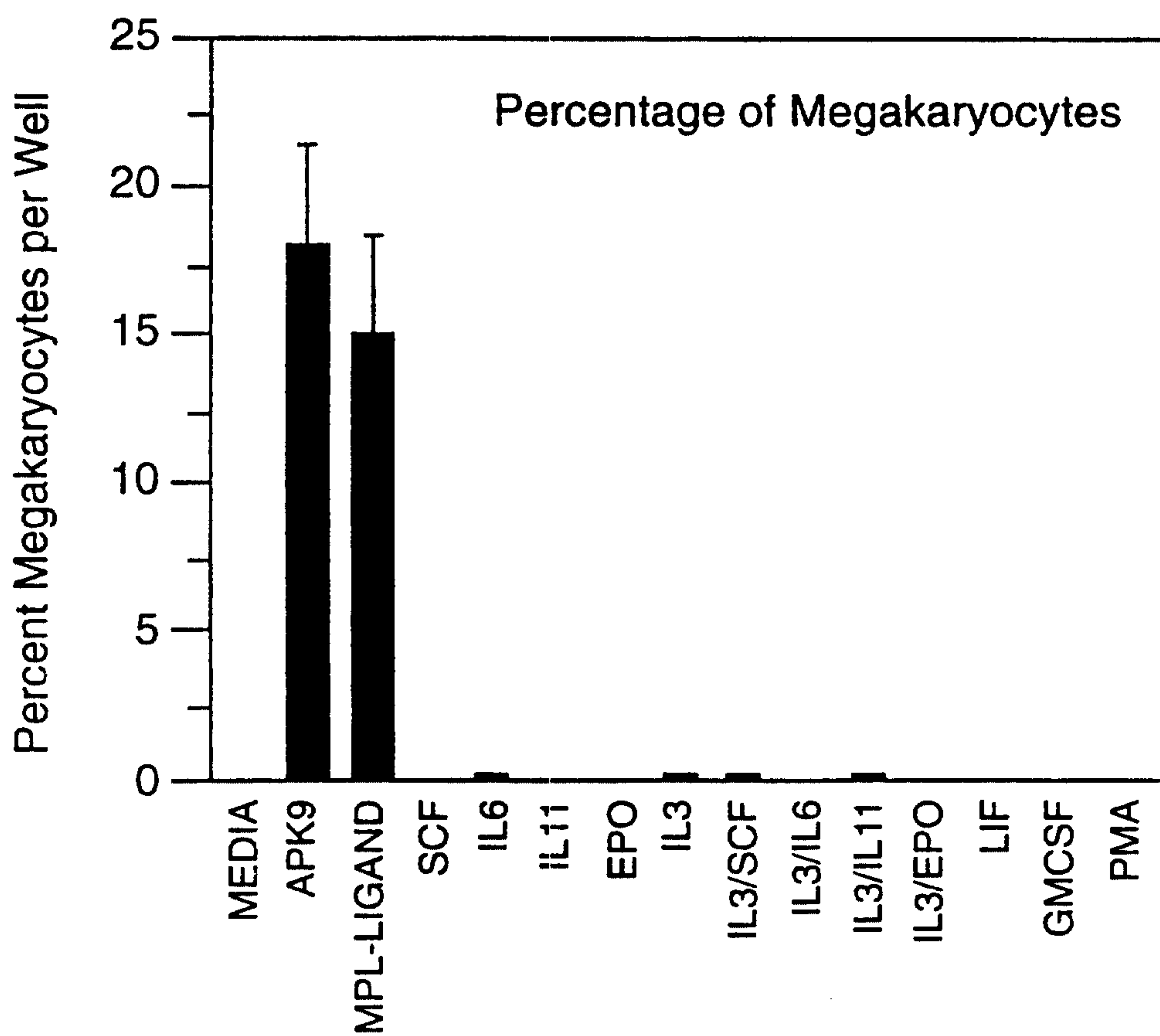


FIG. 8



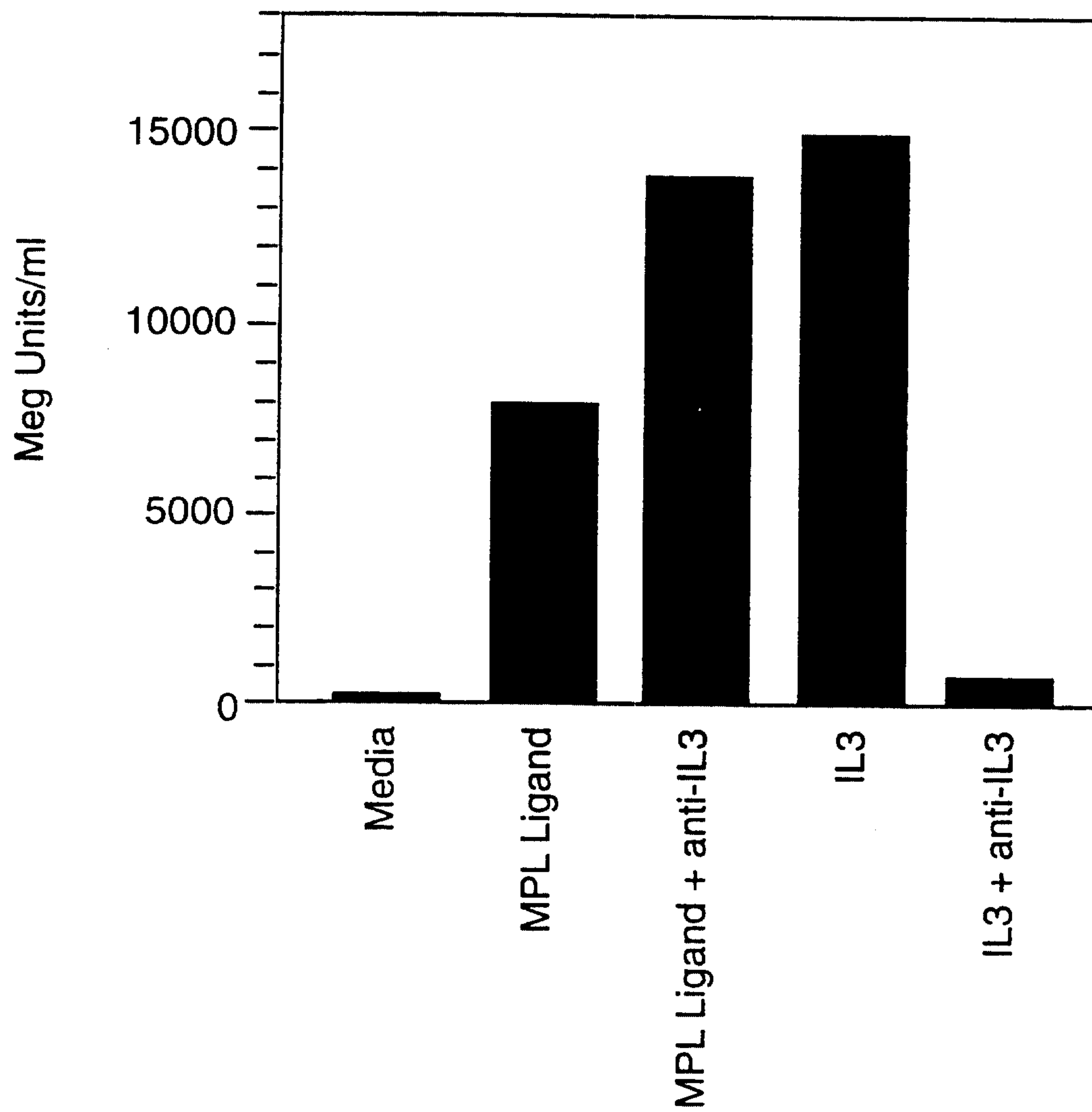
9 / 2 8

FIG. 9



10 / 28

FIG. 10



11 / 28

FIG. 11A

1	CAGGGAGCCACGCCAGCCAAGACACCCCGGCCAGAATGGAGCTGACTGAATTGCTCCTC	59
1	MetGluLeuThrGluLeuLeuLeu	8
60	GTGGTCATGCTTCTCCTAACTGCAAGGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGT	119
9	ValValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCys	28
120	GACCTCCGAGTCCTCAGTAAACTGCTTCGTGACTCCCATGTCCTTCACAGCAGACTGAGC	179
29	AspLeuArgValLeuSerLysLeuLeuArgAspSerHisValLeuHisSerArgLeuSer	48
180	CAGTGCCCGAGAGGTTACCCCTTGCCTACACCTGTCCTGCTGCCTGCTGTGGACTTTAGC	239
49	GlnCysProGluValHisProLeuProThrProValLeuLeuProAlaValAspPheSer	68
240	TTGGGAGAATGGAAAACCCAGATGGAGGAGACCAAGGCACAGGACATTCTGGGAGCAGTG	299
69	LeuGlyGluTrpLysThrGlnMetGluGluThrLysAlaGlnAspIleLeuGlyAlaVal	88
300	ACCCTTCTGCTGGAGGGAGTGATGGCAGCACGGGGACAACCTGGGACCCACTTGCCTCTCA	359
89	ThrLeuLeuLeuGluGlyValMetAlaAlaArgGlyGlnLeuGlyProThrCysLeuSer	108
360	TCCCTCCTGGGGCAGCTTCTGACAGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTC	419
109	SerLeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeu	128
420	CTTGAACCCAGCTTCCTCCACAGGGCAGGACCACAGCTCACAAGGATCCCAATGCCATC	479
129	LeuGlyThrGlnLeuProProGlnGlyArgThrThrAlaHisLysAspProAsnAlaIle	148
480	TTCCTGAGCTTCCAACACCTGCTCCGAGGAAAGGTGCGTTTCCTGATGCTTGTAGGAGGG	539
149	PheLeuSerPheGlnHisLeuLeuArgGlyLysValArgPheLeuMetLeuValGlyGly	168
540	TCCACCCTCTGCGTCAGGCGGGCCCCACCCACCACAGCTGTCCCCAGCAGAACCTCTCTA	599
169	SerThrLeuCysValArgArgAlaProProThrThrAlaValProSerArgThrSerLeu	188
600	GTCCTCACACTGAACGAGCTCCCAAACAGGACTTCTGGATTGTTGGAGACAACTTCACT	659
189	ValLeuThrLeuAsnGluLeuProAsnArgThrSerGlyLeuLeuGluThrAsnPheThr	208
660	GCCTCAGCCAGAACTACTGGCTCTGGGCTTCTGAAGTGGCAGCAGGGATTCAGAGCCAAG	719
209	AlaSerAlaArgThrThrGlySerGlyLeuLeuLysTrpGlnGlnGlyPheArgAlaLys	228
720	ATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTGGACCAAATCCCCGGATACCTGAAC	779
229	IleProGlyLeuLeuAsnGlnThrSerArgSerLeuAspGlnIleProGlyTyrLeuAsn	248
780	AGGATACACGAACTCTTGAATGGAACCTCGTGGACTCTTTCCTGGACCCTCACGCAGGACC	839
249	ArgIleHisGluLeuLeuAsnGlyThrArgGlyLeuPheProGlyProSerArgArgThr	268
840	CTAGGAGCCCCGGACATTTCTCAGGAACATCAGACACAGGCTCCCTGCCACCCAACCTC	899
269	LeuGlyAlaProAspIleSerSerGlyThrSerAspThrGlySerLeuProProAsnLeu	288

FIG. IIB

900	CAGCCTGGATATTCTCCTTCCCCAACCCATCCTCCTACTGGACAGTATACGCTCTTCCCT	959
289	GlnProGlyTyrSerProSerProThrHisProProThrGlyGlnTyrThrLeuPhePro	308
960	CTTCCACCCACCTTGCCCACCCCTGTGGTCCAGCTCCACCCCCTGCTTCCTGACCCTTCT	1019
309	LeuProProThrLeuProThrProValValGlnLeuHisProLeuLeuProAspProSer	328
1020	GCTCCAACGCCCACCCCTACCAGCCCTCTTCTAAACACATCCTACACCCACTCCCAGAAT	1079
329	AlaProThrProThrProThrSerProLeuLeuAsnThrSerTyrThrHisSerGlnAsn	348
1080	CTGTCTCAGGAAGGGTAAGGTTCTCAGACACTGCCGACATCAGCATTGTCTCGTGTACAG	1139
349	LeuSerGlnGluGlyEnd	353
1140	CTCCCTTCCCTGCAGGGCGCCCCTGGGAGACAACCTGGACAAGATTTCTACTTTCTCCTG	1199
1200	AAACCCAAAGCCCTGGTAAAAGGGATACACAGGACTGAAAAGGGAATCATTTTTCACTGT	1259
1260	ACATTATAAACCTTCAGAAGCTATTTTTTTAAGCTATCAGCAATACTCATCAGAGCAGCT	1319
1320	AGCTCTTTGGTCTATTTTCTGCA	1342

13 / 28

FIG. 12A

1	AGGGAGCCACGCCAGCCAGACACCCCGGCCAGAATGGAGCTGACTGAATTGCTCCTCGTG	60
	MetGluLeuThrGluLeuLeuLeuVal	9
61	GTCATGCTTCTCCTAACTGCAAGGCTAACGCTGTCCAGCCCGGCTCCTCCTGCTTGTGAC	120
10	ValMetLeuLeuLeuThrAlaArgLeuThrLeuSerSerProAlaProProAlaCysAsp	29
121	CTCCGAGTCCTCAGTAAACTGCTTCGTGACTCCCATGTCCTTCACAGCAGACTGAGCCAG	180
30	LeuArgValLeuSerLysLeuLeuArgAspSerHisValLeuHisSerArgLeuSerGln	49
181	TGCCCAGAGGTTACCCCTTTGCCTACACCTGTCCTGCTGCCTGCTGTGGACTTTAGCTTG	240
50	CysProGluValHisProLeuProThrProValLeuLeuProAlaValAspPheSerLeu	69
241	GGAGAATGGAAAACCCAGATGGAGGAGACCAAGGCACAGGACATTCTGGGAGCAGTGACC	300
70	GlyGluTrpLysThrGlnMetGluGluThrLysAlaGlnAspIleLeuGlyAlaValThr	89
301	CTTCTGCTGGAGGGAGTGATGGCAGCACGGGGACAACCTGGGACCCACTTGCCTCTCATCC	360
90	LeuLeuLeuGluGlyValMetAlaAlaArgGlyGlnLeuGlyProThrCysLeuSerSer	109
361	CTCCTGGGGCAGCTTTCTGGACAGGTCCGTCTCCTCCTTGGGGCCCTGCAGAGCCTCCTT	420
110	LeuLeuGlyGlnLeuSerGlyGlnValArgLeuLeuLeuGlyAlaLeuGlnSerLeuLeu	129
421	GGAACCCAGCTTCCTCCACAGGGCAGGACCACAGCTCACAAGGATCCCAATGCCATCTTC	480
130	GlyThrGlnLeuProProGlnGlyArgThrThrAlaHisLysAspProAsnAlaIlePhe	149
481	CTGAGCTTCCAACACCTGCTCCGAGGAAAGGACTTCTGGATTGTTGGAGACAAACTTCAC	540
150	LeuSerPheGlnHisLeuLeuArgGlyLysAspPheTrpIleValGlyAspLysLeuHis	169
541	TGCCTCAGCCAGAACTACTGGCTCTGGGCTTCTGAAGTGGCAGCAGGGATTTCAGAGCCAA	600
170	CysLeuSerGlnAsnTyrTrpLeuTrpAlaSerGluValAlaAlaGlyIleGlnSerGln	189
601	GATTCCTGGTCTGCTGAACCAAACCTCCAGGTCCCTGGACCAAATCCCCGGATACCTGAA	660
190	AspSerTrpSerAlaGluProAsnLeuGlnValProGlyProAsnProArgIleProGlu	209
661	CAGGATACACGAACTCTTGAATGGAACCTCGTGGACTCTTTCCTGGACCCTCACGCAGGAC	720
210	GlnAspThrArgThrLeuGluTrpAsnSerTrpThrLeuSerTrpThrLeuThrGlnAsp	229
721	CCTAGGAGCCCCGGACATTTCTCCTCAGGAACATCAGACACAGGCTCCCTGCCACCCAACCT	780
230	ProArgSerProGlyHisPheLeuArgAsnIleArgHisArgLeuProAlaThrGlnPro	249
781	CCAGCCTGGATATTCTCCTTCCCCAACCCTCCTCCTACTGGACAGTATACGCTCTTCCC	840
250	ProAlaTrpIlePheSerPheProAsnProSerSerTyrTrpThrValTyrAlaLeuPro	269

2167090

14 / 28

FIG. 12 B

841	TCTTCCACCCACCTTGCCCCACCCCTGTGGTCCAGCTCCACCCCTGCTTCCTGACCCTTC	900
270	SerSerThrHisLeuAlaHisProCysGlyProAlaProProProAlaSerEnd	286
901	TGCTCCAACGCCCACCCCTACCAGCCCTCTTCTAAACACATCCTACACCCACTCCCAGAA	960
961	TCTGTCTCAGGAAGGGTAAGGTTCTCAGACACTGCCGACATCAGCATTGTCTCGTGTACA	1020
1021	GCTCCCTTCCCTGCAGGGCGCCCCTGGGAGACAAGTTTCCTACTTTCTCCT	1080
1081	GAAACCCAAAGCCCTGGTAAAAGGGATACACAGGACTGAAAAGGGAATCATTTTTCACTG	1140
1141	TACATTATAAACCTTCAGAAGCTA	1164

15 / 28

FIG. 13A

```
canine 1 MELTELLLVMLLLTARLDPCLPAPPACDPRLLNKMLRDSHVLHSRLSQC 50
          |||||
human 1 MELTELLLVMLLLTARLTLSPPAPPACDLRVLSKLLRDSHVLHSRLSQC 50
          |||||

51 PDIYPLSTPVLLPAVDFSLGEWKTQKEQTKAQDVWGAVALLLDGVLAARG 100
   |:|.|||
51 PEVHPLPTPVLLPAVDFSLGEWKTQMEETKAQDILGAVTLLLEGVMAARG 100
   |:|.|||

101 QLGPSCLSSLLGQLSGQVRLLLGALQGLLGTQLPPQGRTTTHKDPNAIFL 150
    |||.|||
101 QLGPTCLSSLLGQLSGQVRLLLGALQSLTGTQLPPQGRTTAHKDPNAIFL 150
    |||.|||

151 SFQQLLRGKVRFLLLVAGPTLCAKQSQPTTAVPTNTSLFLTLRKLPNRTS 200
    |||:|||||
151 SFQHLLRGKVRFLMLVGGSTLCVRRAPPTTAVPSRTSLVLTNLNLPNRTS 200
    |||:|||||

201 GLETNSSISARTTGSGLLKRLQGFRAKIPGLLNQTSRSLNQTGHLNRT 250
    |||||.|||
201 GLETNFTASARTTGSGLLKRWQGFRAKIPGLLNQTSRSLDQIPGYLNRI 250
    |||||.|||

251 HGPLNGTHGLLPGLSLTALGAPDIPPGTSDMDALPPNLWPRYSPSPHPP 300
    |:| |||:| |||.|||
251 HELNGTRGLFPGPSRRTLGAPEISSGTSDTGSLLPPLNLPQGYSPSPHPP 300
    |:| |||:| |||.|||

301 PGQYTLFSPPLPTSPTPQNPLQPPPPDPSA.TANSTSPLLIAAHPHFQNLS 349
    .||| |||.|||
301 TGQYTLFPLPPTLPTPVVQLHPLLPDPSAPTPTPTSPLLNTSYTHSQNLS 350
    .||| |||.|||

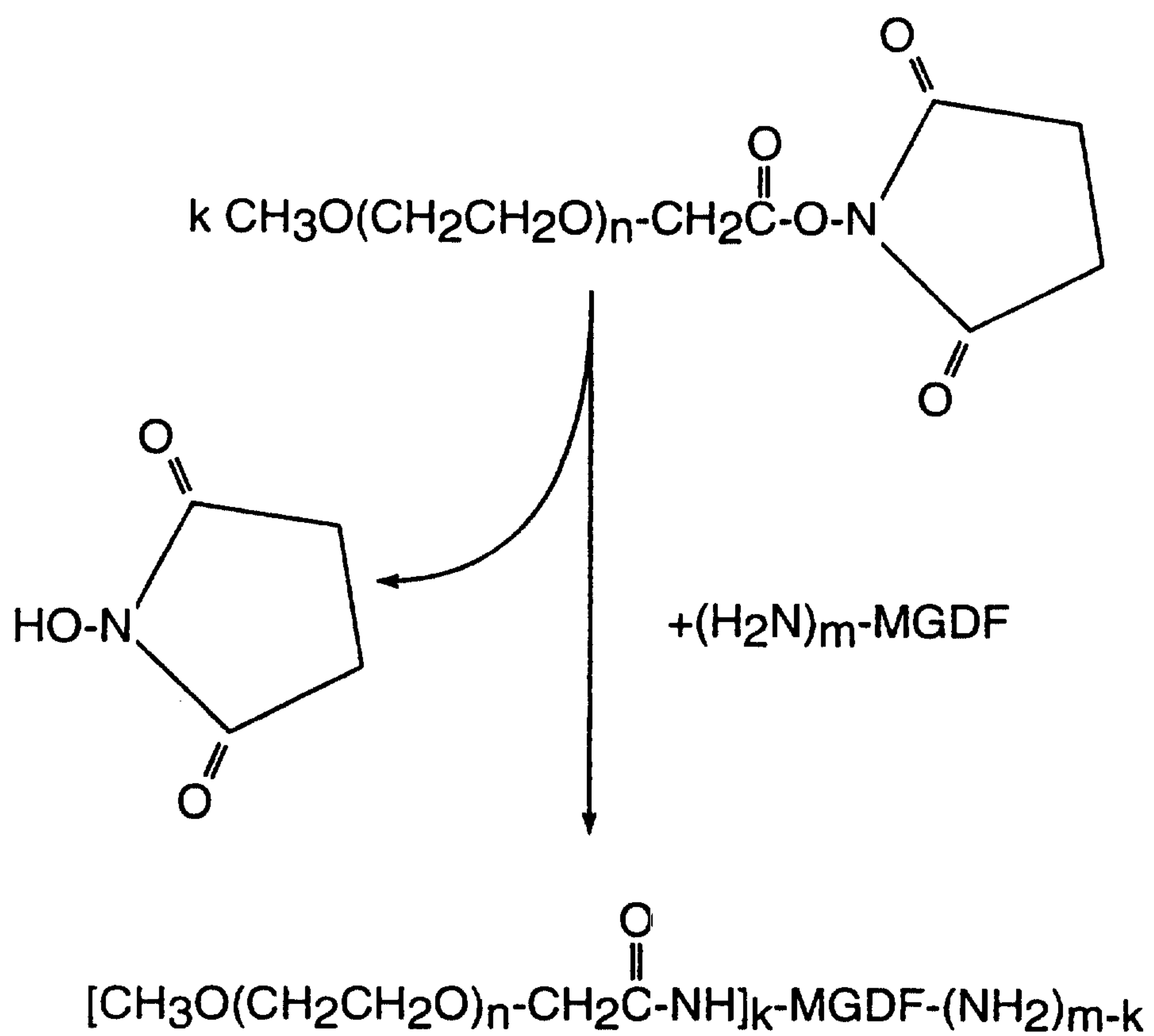
350 QEE 352
    ||:
351 QEG 353
```

FIG. 13B

[illegible]

17 / 28

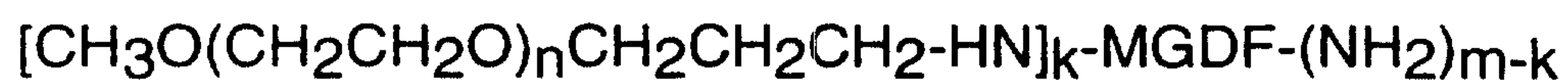
FIG. 14



k , m and n are the same as defined in Figure 15.

18 / 28

FIG. 15


$$\downarrow \text{NaCNBH}_3$$


- k - number of PEG molecules reacted with a molecule of MGDF.
- n - degree of polymerization of PEG used in the reaction; e.g. n=2000 for PEG of MW=100 kD; n=40 for PEG of MW=2 kD.
- m - total number of primary amino groups per MGDF molecule.

19 / 28

FIG. 16

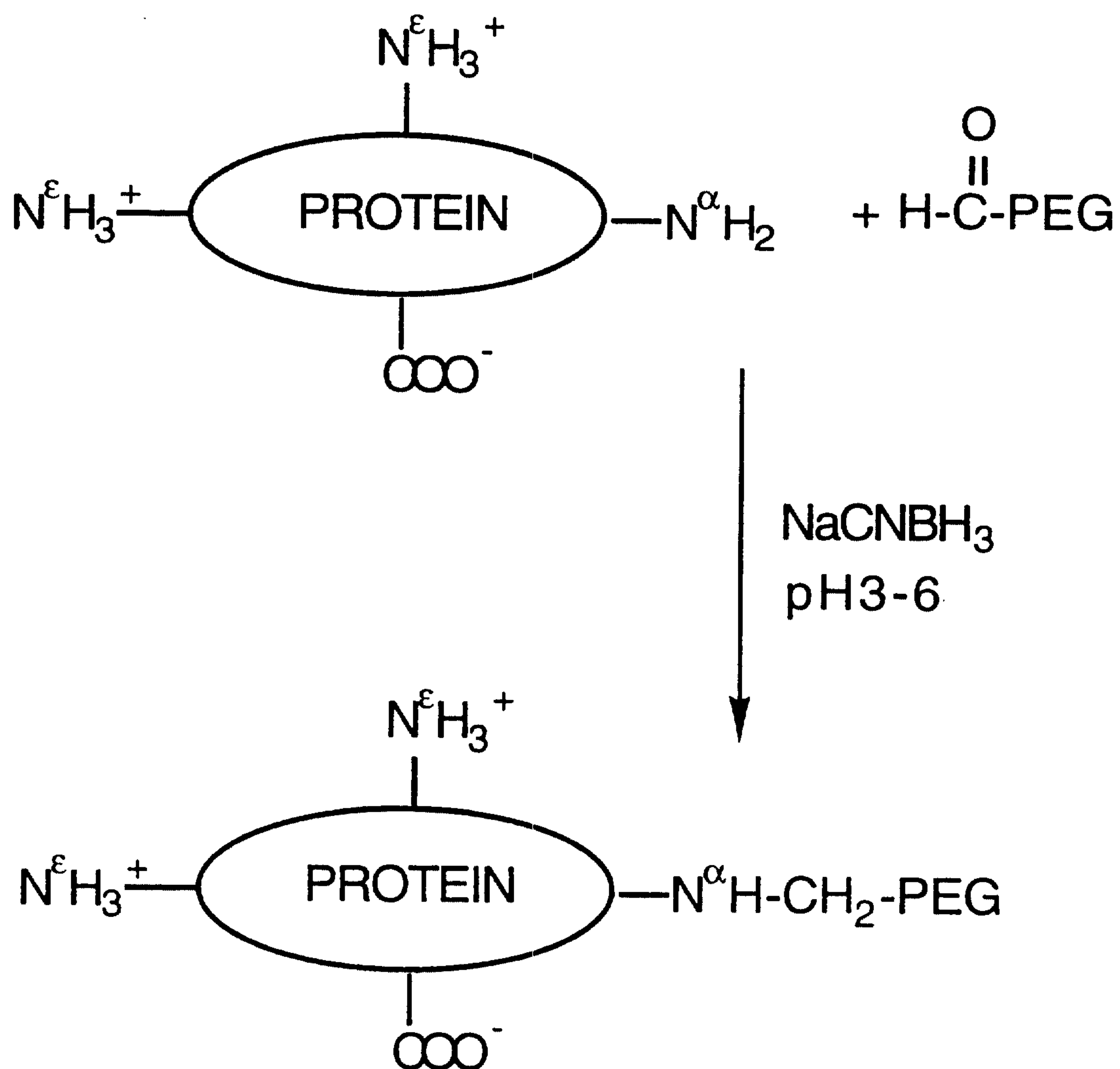


FIG. 17A

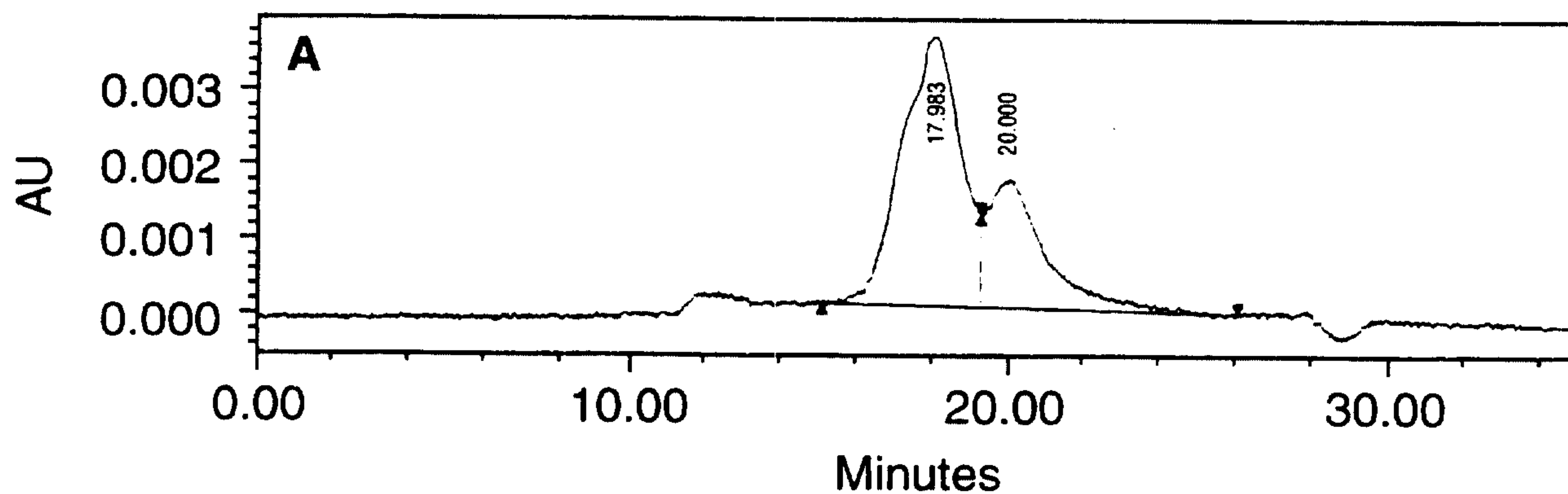


FIG. 17B

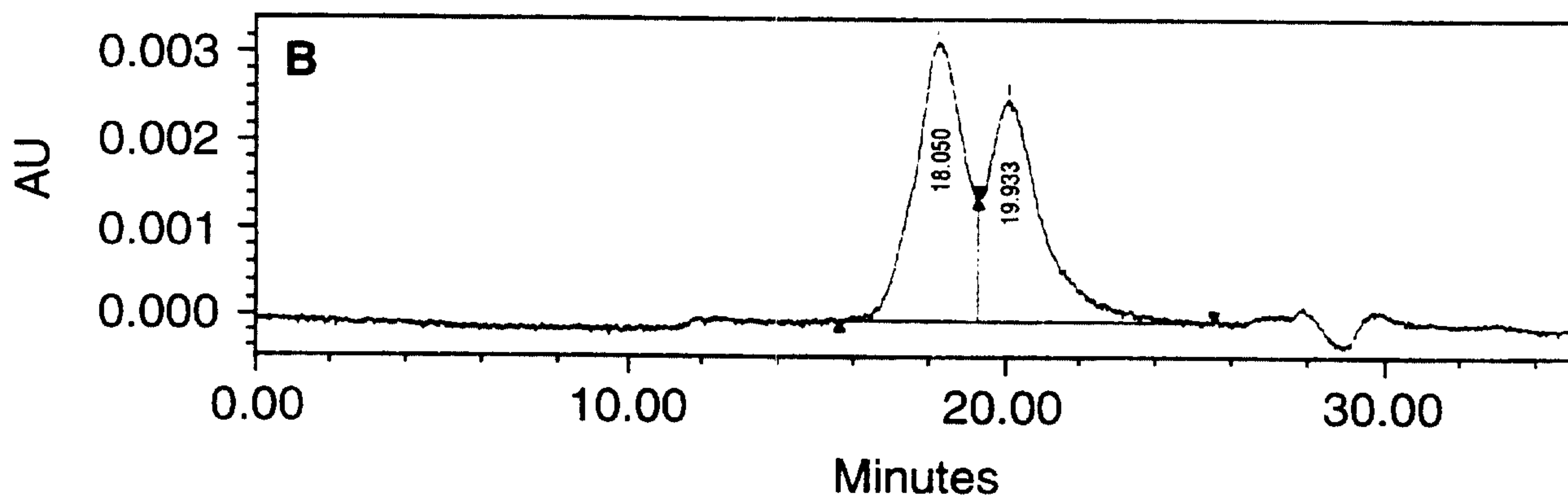


FIG. 17C

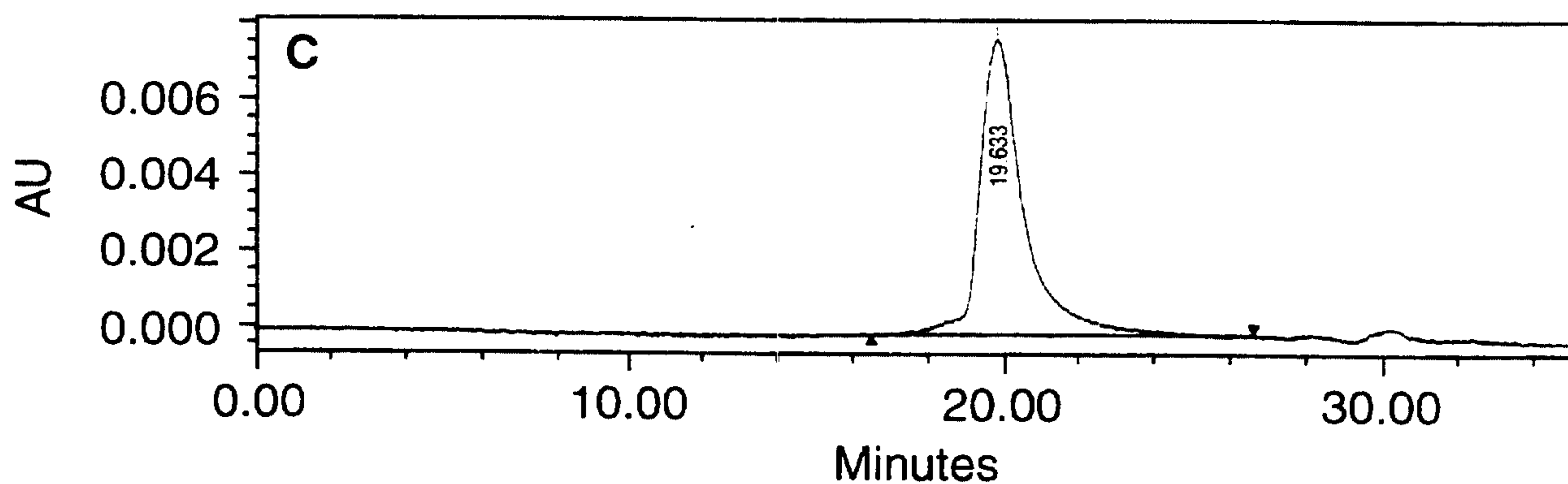


FIG. 18

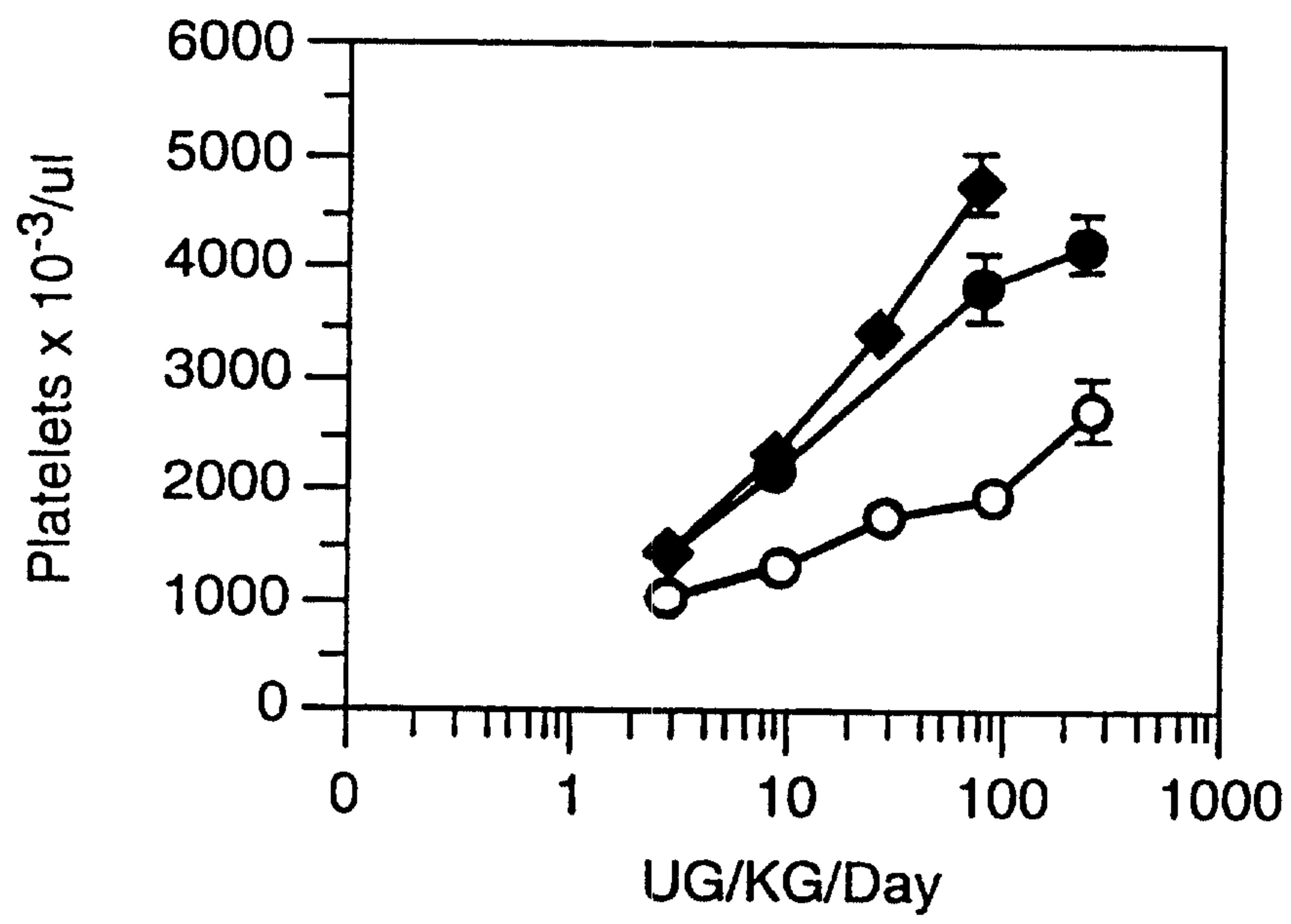


FIG. 19

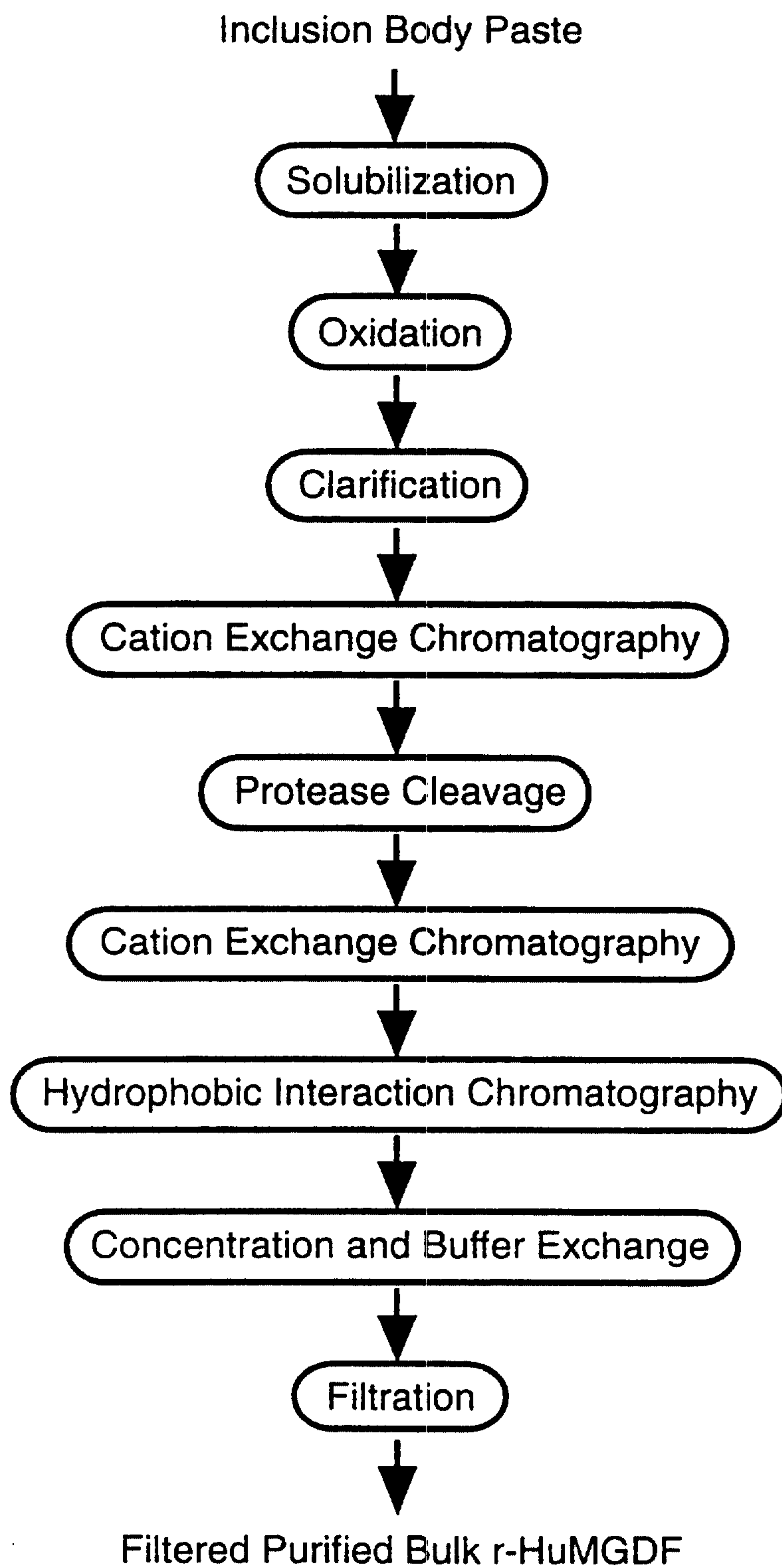


FIG. 20

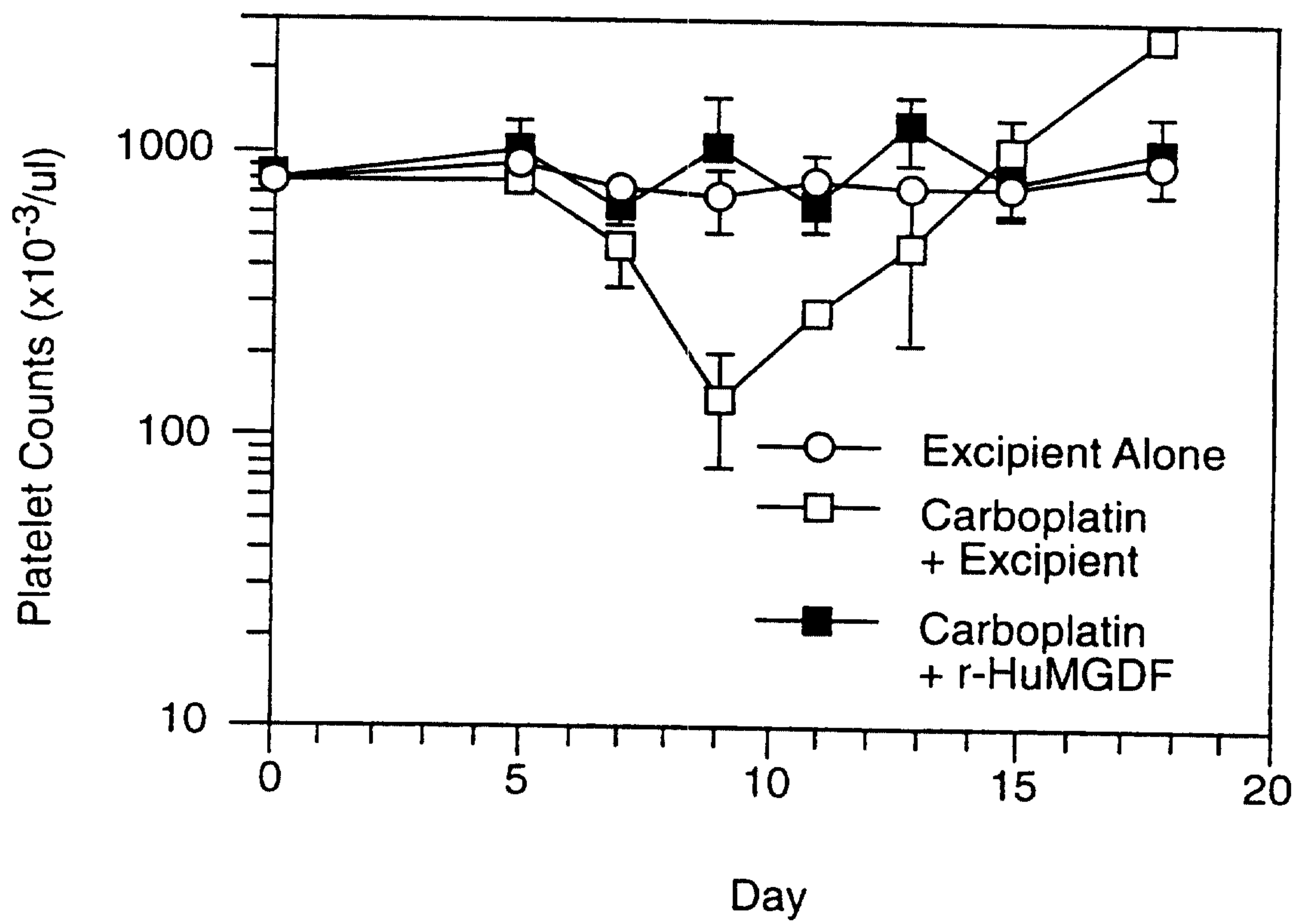


FIG. 21

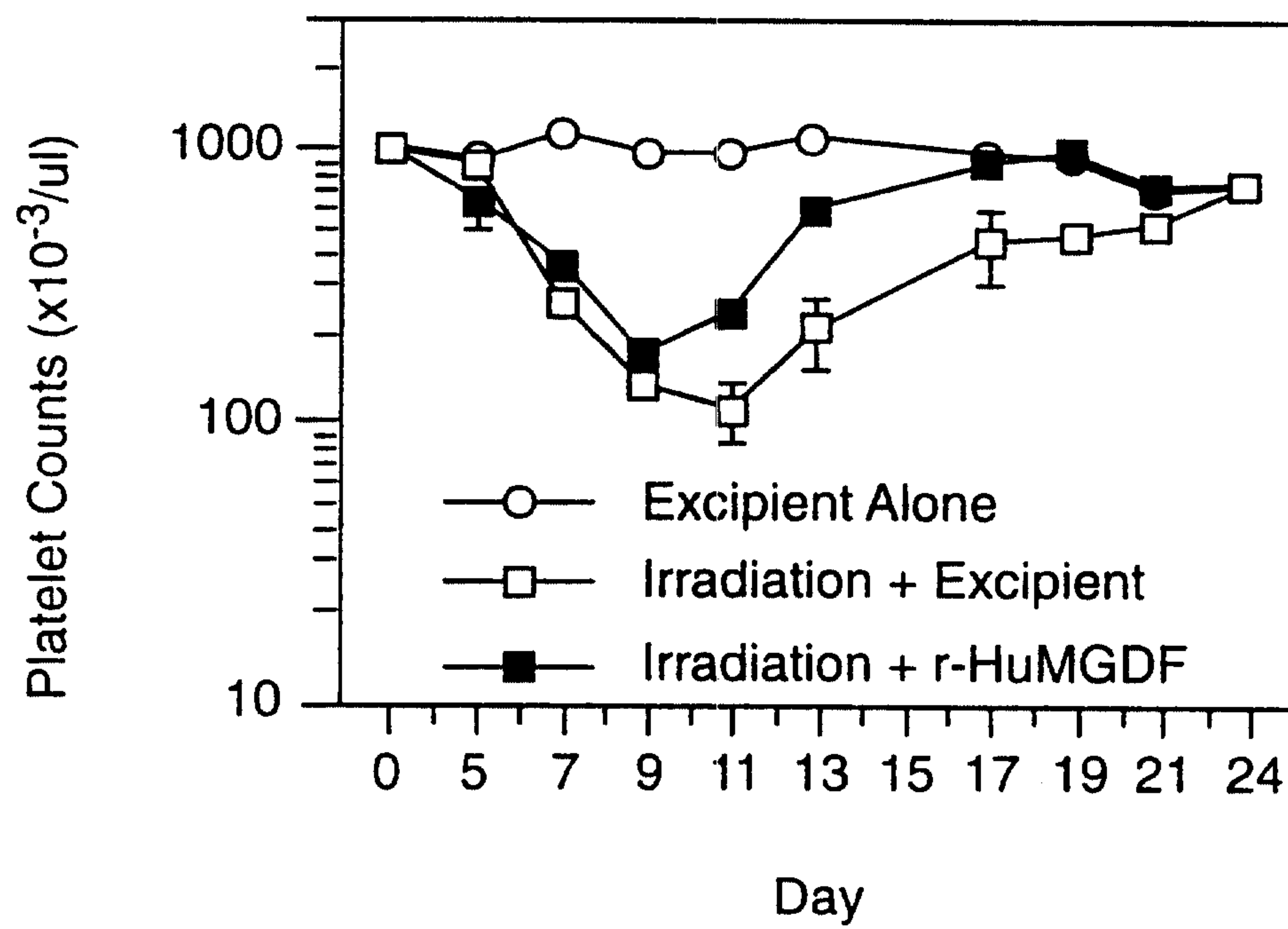
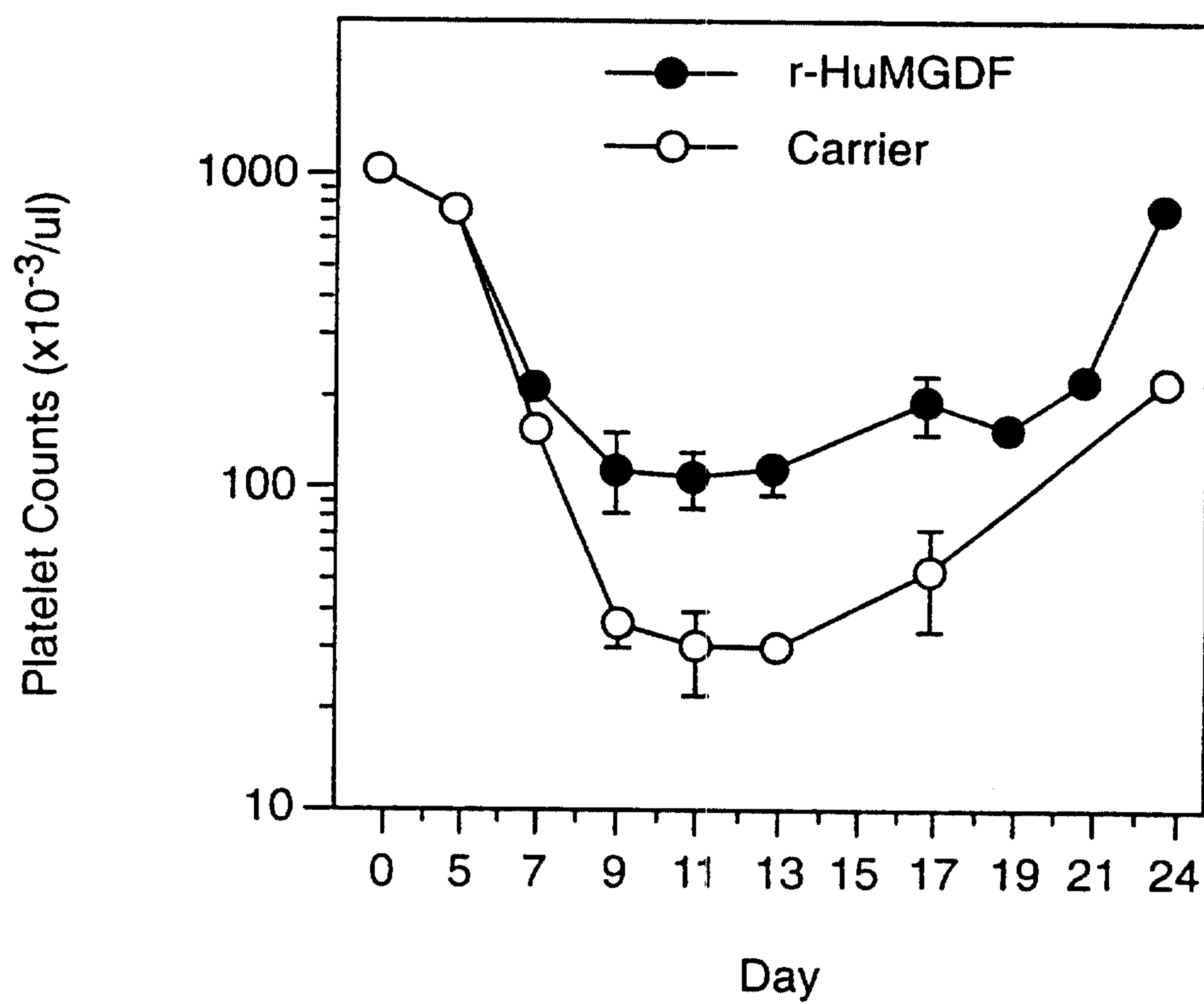
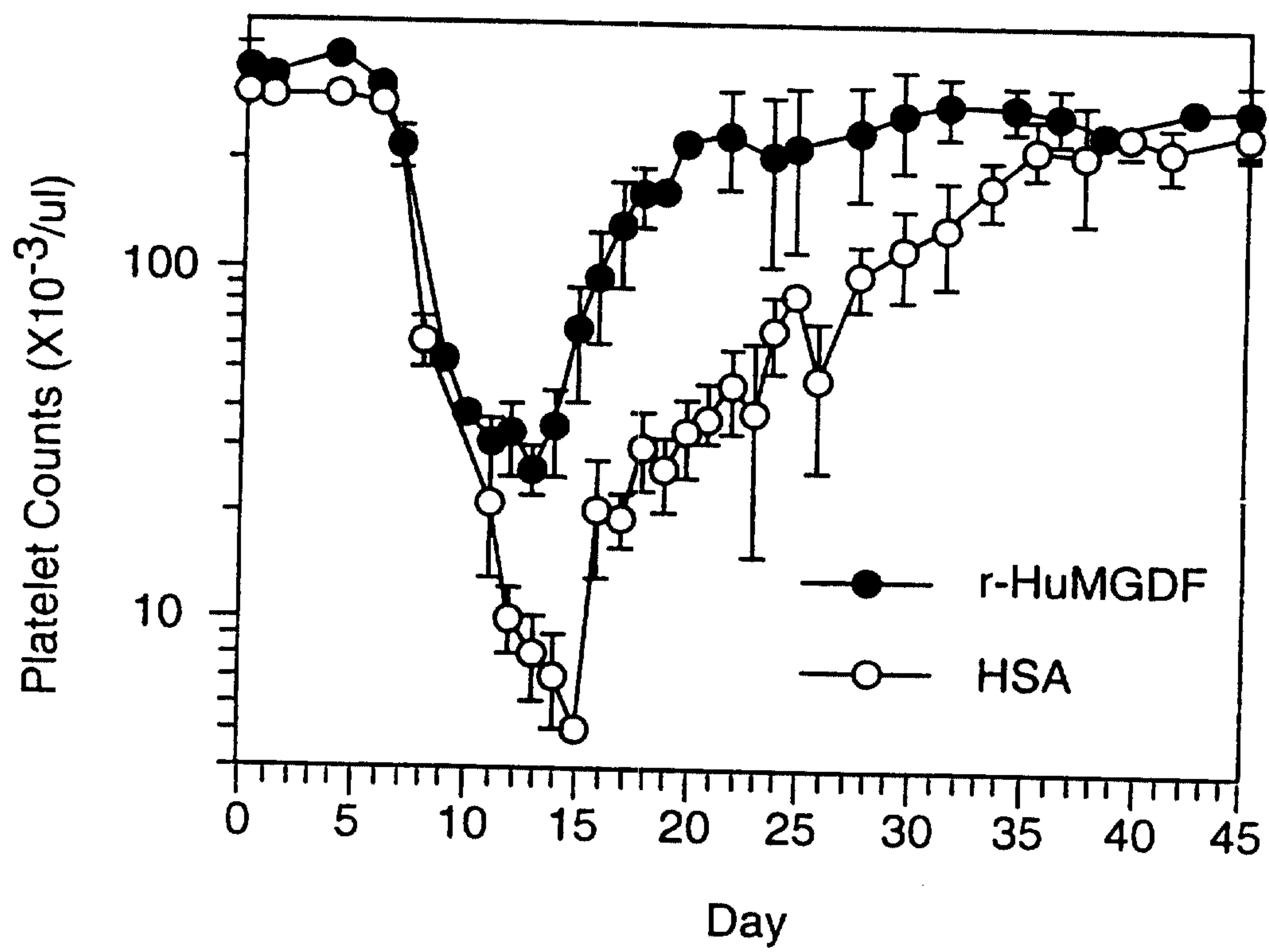


FIG. 22



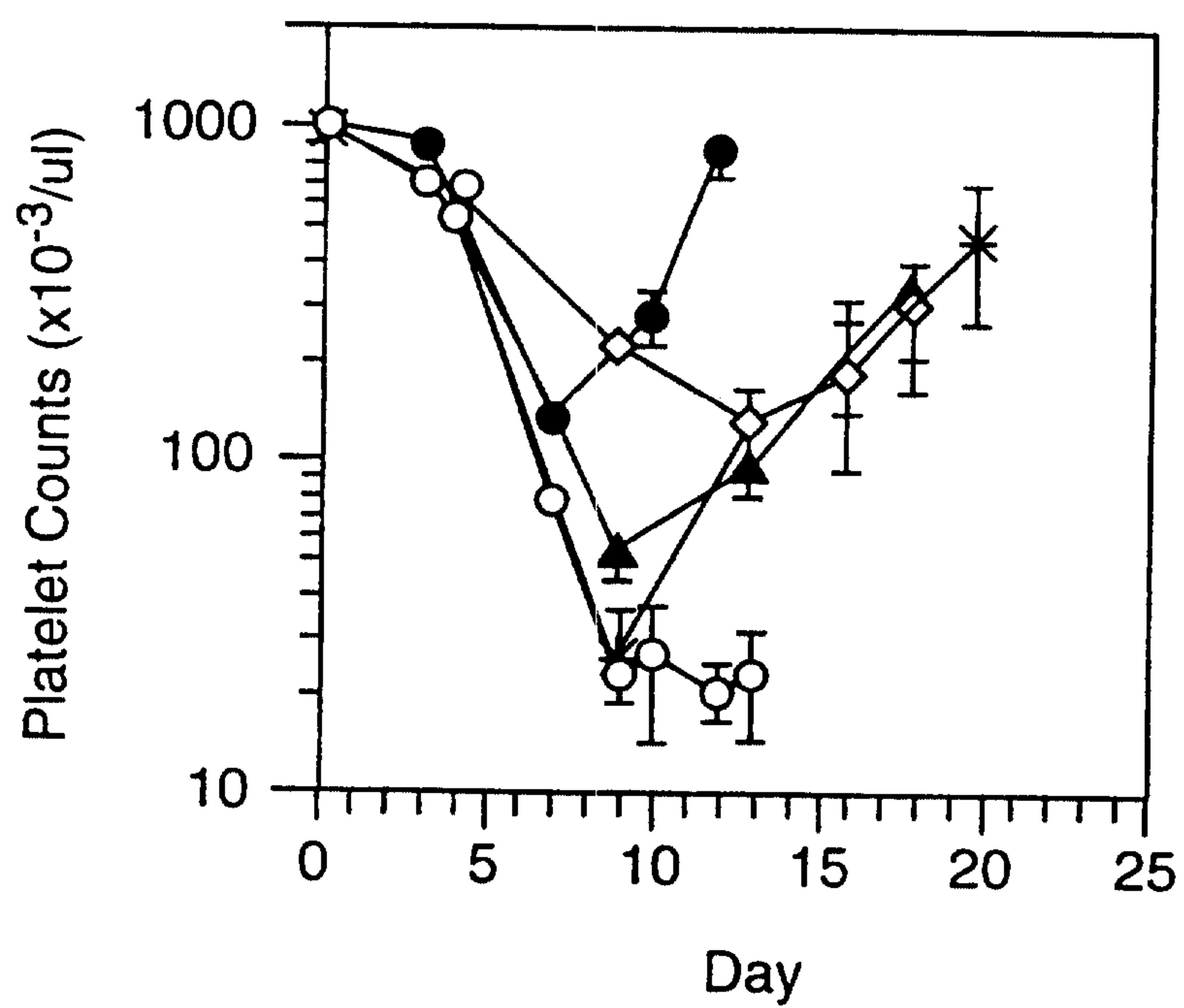
26 / 28

FIG. 23



27/28

FIG. 24



- coli 1-163 (12kd dipeg) (PEG 22)
- ◇— CHO 1-332
- ▲— coli 1-163 (25kd monopeg) (PEG 17)
- *— coli 1-163 (20kd monopeg) (PEG16)
- ctl

28 / 28
FIG. 25

ATG AAA AGT CCT GCA CCA CCT GCA TGT GAT TTA CGG GTC CTG
MET LYS SER PRO ALA PRO PRO ALA CYS ASP LEU ARG VAL LEU

TCT AAA CTG CTG CGC GAC TCT CAC GTG CTG CAC TCT CGT CTG
SER LYS LEU LEU ARG ASP SER HIS VAL LEU HIS SER ARG LEU

TCC CAG TGC CCG GAA GTT CAC CCG CTG CCG ACC CCG GTT CTG
SER GLN CYS PRO GLU VAL HIS PRO LEU PRO THR PRO VAL LEU

CTT CCG GCT GTC GAC TTC TCC CTG GGT GAA TGG AAA ACC CAG
LEU PRO ALA VAL ASP PHE SER LEU GLY GLU TRP LYS THR GLN

ATG GAA GAG ACC AAA GCT CAG GAC ATC CTG GGT GCA GTA ACT
MET GLU GLU THR LYS ALA GLN ASP ILE LEU GLY ALA VAL THR

CTG CTT CTG GAA GGC GTT ATG GCT GCA CGT GGC CAG CTT GGC
LEU LEU LEU GLU GLY VAL MET ALA ALA ARG GLY GLN LEU GLY

CCG ACC TGC CTG TCT TCC CTG CTT GGC CAG CTG TCT GGC CAG
PRO THR CYS LEU SER SER LEU LEU GLY GLN LEU SER GLY GLN

GTT CGT CTG CTG CTC GGC GCT CTG CAG TCT CTG CTT GGC ACC
VAL ARG LEU LEU LEU GLY ALA LEU GLN SER LEU LEU GLY THR

CAG CTG CCG CCA CAG GGC CGT ACC ACT GCT CAC AAG GAT CCG
GLN LEU PRO PRO GLN GLY ARG THR THR ALA HIS LYS ASP PRO

AAC GCT ATC TTC CTG TCT TTC CAG CAC CTG CTG CGT GGC AAA
ASN ALA ILE PHE LEU SER PHE GLN HIS LEU LEU ARG GLY LYS

GTT CGT TTC CTG ATG CTG GTT GGC GGT TCT ACC CTG TGC GTT
VAL ARG PHE LEU MET LEU VAL GLY GLY SER THR LEU CYS VAL

CGT CGG GCG CCG CCA ACC ACT GCT GTT CCG TCT TAA
ARG ARG ALA PRO PRO THR THR ALA VAL PRO SER STOP