



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/27, C12P 21/02 C07K 13/00, C12N 15/12 G01N 33/68	A1	(11) International Publication Number: WO 93/25687 (43) International Publication Date: 23 December 1993 (23.12.93)
(21) International Application Number: PCT/US93/05548 (22) International Filing Date: 9 June 1993 (09.06.93) (30) Priority data: 07/896,512 9 June 1992 (09.06.92) US (60) Parent Application or Grant (63) Related by Continuation US 07/896,512 (CIP) Filed on 9 June 1992 (09.06.92) (71) Applicants (for all designated States except US): CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US). THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive 2nd Floor, Oakland, CA 94612-3550 (US). (72) Inventors; and (75) Inventors/Applicants (for US only) : PANDIT, Jayvardhan [IN/US]; 12 Water Street Apt 114, Mystic, CT 06355 (US). JANCARIK, Jarmila [US/US]; 1954 Woodbury Court, Walnut Creek, CA 94596 (US). KIM, Sung-Hou [US/US]; 1080 Country Club Drive, Moraga, CA 94556 (US). KOTHS, Kirston [US/US]; 2646 Mira Vista Drive, El Cerrito, CA 94530 (US). HALENBECK, Robert [US/US]; 136 Spring Grove Avenue, San Rafael, CA 94901		(US). FEAR, Anna, Lisa [US/US]; 4418 Webster Street, Oakland, CA 94609 (US). TAYLOR, Eric [US/US]; 561 62nd Street, Oakland, CA 94609 (US). YAMAMOTO, Ralph [US/US]; 423 Pine Bark Court, Martinez, CA 94553 (US). BOHM, Andrew [US/US]; 2431 Elsworth Apt 203, Berkeley, CA 94704 (US). (74) Agent: GRUBER, Lewis, S.; Marshall, O'Toole, Gerstein, Murray & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606-6402 (US). (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: CRYSTALLIZATION OF M-CSF (57) Abstract <p>The present invention is directed to methods for crystallizing macrophage colony stimulating factor. The present invention is also directed to methods for designing and producing M-CSF agonists and antagonists using information derived from the crystallographic structure of M-CSF. The invention is also directed to methods for screening M-CSF agonists and antagonists. In addition, the present invention is directed to an isolated, purified, soluble and functional M-CSF receptor.</p>		

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CRYSTALLIZATION OF M-CSF

Work described herein was funded with Government support.

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This application is a continuation-in-part of U.S. Serial No. 07/896,512

5 filed June 9, 1992.

FIELD OF THE INVENTION

The present invention relates in general to crystalline compositions of macrophage colony stimulating factor "M-CSF" and in particular to methods for the use of structural information (including X-ray diffraction patterns) of crystalline M-
10 CSF for agonist and antagonist production, as well as assays for detection of same.

BACKGROUND OF THE INVENTION

Monocyte-macrophage colony-stimulating factor is produced by a variety of cells, including macrophages, endothelial cells and fibroblasts (*see*, Ralph *et al.*, "The Molecular and Biological Properties of the Human and Murine Members
15 of the CSF-1 Family" in *Molecular Basis of Lymphokine Action*, Humana Press, Inc., (1987), which is incorporated herein by reference). M-CSF is composed of two "monomer" polypeptides, which form a biologically active dimeric M-CSF protein (hereinafter referred to as "M-CSF dimer"). M-CSF belongs to a group of biological agonists that promote the production of blood cells. Specifically, it acts as a growth
20 and differentiation factor for bone marrow progenitor cells of the mononuclear phagocyte lineage. Further, M-CSF stimulates the proliferation and function of mature macrophages via specific receptors on responding cells. In clinical trials M-CSF has shown promise as a pharmaceutical agent in the correction of blood cell deficiencies arising as a side-effect of chemotherapy or radiation therapy for cancer
25 and may be beneficial in treating fungal infections associated with bone marrow transplants. M-CSF may also play significant biological roles in pregnancy, uveitis,

and atherosclerosis. Development of M-CSF agonists or antagonists may prove to be of value in modifying the biological events involved in these conditions.

M-CSF exists in at least three mature forms: short (M-CSF α), intermediate (M-CSF γ), and long (M-CSF β). Mature M-CSF is defined as including polypeptide sequences contained within secreted M-CSF following amino terminus processing to remove leader sequences and carboxyl terminus processing to remove domains including a putative transmembrane region. The variations in the three mature forms are due to alternative mRNA splicing (*see, Cerretti et al. Molecular Immunology, 25:761 (1988)*). The three forms of M-CSF are translated from different mRNA precursors, which encode polypeptide monomers of 256 to 554 amino acids, having a 32 amino acid signal sequence at the amino terminal and a putative transmembrane region of approximately 23 amino acids near the carboxyl terminal. The precursor peptides are subsequently processed by amino terminal and carboxyl terminal proteolytic cleavages to release mature M-CSF. Residues 1-149 of all three mature forms of M-CSF are identical and are believed to contain sequences essential for biological activity of M-CSF. *In vivo* M-CSF monomers are dimerized via disulfide-linkage and are glycosylated. Some, if not all, forms of M-CSF can be recovered in membrane-associated form. Such membrane-bound M-CSF may be cleaved to release secreted M-CSF. Membrane associated M-CSF is believed to have biological activity similar to M-CSF, but may have other activities including cell-cell association or activation.

Polypeptides, including the M-CSFs, have a three-dimensional structure determined by the primary amino acid sequence and the environment surrounding the polypeptide. This three-dimensional structure establishes the polypeptide's activity, stability, binding affinity, binding specificity, and other biochemical attributes. Thus, a knowledge of a protein's three-dimensional structure can provide much guidance in designing agents that mimic, inhibit, or improve its biological activity in soluble or membrane bound forms.

The three-dimensional structure of a polypeptide may be determined in a number of ways. Many of the most precise methods employ X-ray

crystallography (for a general review, see, Van Holde, *Physical Biochemistry, Prentice-Hall, N.J.* pp. 221-239, (1971), which is incorporated herein by reference). This technique relies on the ability of crystalline lattices to diffract X-rays or other forms of radiation. Diffraction experiments suitable for determining the three-dimensional structure of macromolecules typically require high-quality crystals. Unfortunately, such crystals have been unavailable for M-CSF as well as many other proteins of interest. Thus, high-quality, diffracting crystals of M-CSF would assist the determination of its three-dimensional structure.

Various methods for preparing crystalline proteins and polypeptides are known in the art (*see, for example, McPherson, et al. "Preparation and Analysis of Protein Crystals", A. McPherson, Robert E. Krieger Publishing Company, Malabar, Florida (1989); Weber, Advances in Protein Chemistry 41:1-36 (1991); U.S. Patent No. 4,672,108; and U.S. Patent No. 4,833,233; all of which are incorporated herein by reference for all purposes*). Although there are multiple approaches to crystallizing polypeptides, no single set of conditions provides a reasonable expectation of success, especially when the crystals must be suitable for X-ray diffraction studies. Thus, in spite of significant research, many proteins remain uncrystallized.

In addition to providing structural information, crystalline polypeptides provide other advantages. For example, the crystallization process itself further purifies the polypeptide, and satisfies one of the classical criteria for homogeneity. In fact, crystallization frequently provides unparalleled purification quality, removing impurities that are not removed by other purification methods such as HPLC, dialysis, conventional column chromatography, etc. Moreover, crystalline polypeptides are often stable at ambient temperatures and free of protease contamination and other degradation associated with solution storage. Crystalline polypeptides may also be useful as pharmaceutical preparations. Finally, crystallization techniques in general are largely free of problems such as denaturation associated with other stabilization methods (e.g. lyophilization). Thus, there exists a significant need for preparing M-CSF compositions in crystalline form and determining their three-dimensional

structure. The present invention fulfills this and other needs. Once crystallization has been accomplished, crystallographic data provides useful structural information which may assist the design of peptides that may serve as agonists or antagonists. In addition, the crystal structure provides information useful to map, the receptor
5 binding domain which could then be mimicked by a small non-peptide molecule which may serve as an antagonist or agonist.

SUMMARY OF THE INVENTION

The present invention provides crystalline forms of M-CSF dimers. Preferably, the dimers are formed from polypeptides containing between 146 to 162
10 amino acids residues at or near the N-terminus of mature M-CSF (e.g. glu₁ glu₂ val₃...). In a specific embodiment, the polypeptide includes residues 4 to 158 of mature M-CSF α polypeptide, preferably in the non-glycosylated form.

Another aspect of the invention provides a method of crystallizing an M-CSF. A preferred crystallization method according to the present invention
15 includes the following steps: mixing a preselected, substantially pure M-CSF dimer and a precipitant to form an M-CSF mixture; precipitating crystalline M-CSF from the mixture; and isolating the M-CSF in crystalline form. In some specific embodiments, the precipitant contains polyethylene glycol. Other components such as ammonium sulfate and/or other ionic compounds may be added to the solution.
20 It has been found by x-ray crystallography that M-CSF produced by the method of the present invention can crystallize into the P2₁2₁2₁ space groups for example.

Variations of the crystallization method are also provided. For example, the step of precipitating crystals from the M-CSF mixture may involve equilibrating the M-CSF mixture with a second mixture. The second mixture is
25 typically a solution that consists of a higher concentration of precipitant than the first M-CSF mixture. The step of equilibrating preferably consists of applying the M-CSF mixture to a surface and allowing the applied M-CSF mixture to come into equilibrium with a reservoir of the second mixture. In other embodiments, the step of precipitating M-CSF crystals is initiated by seeding the M-CSF mixture with seed

crystals or altering the temperature of the M-CSF mixture. Another aspect of the invention involves identifying compounds that have structures that mimic a receptor binding region of the three-dimensional structure of M-CSF to varying degrees and can in many instances function as M-CSF agonists or antagonists. Compounds that
5 interact with the receptor-binding region of M-CSF may be antagonists. The three-dimensional alpha-carbon coordinates of a truncated M-CSF dimer is presented in Appendix 1. In one embodiment of the present invention, the three-dimensional structure of M-CSF is obtained by first crystallizing an M-CSF dimer (having M-CSF receptor-binding residues) to form at least one M-CSF crystal. Next, a source of
10 radiation is used for irradiating an M-CSF crystal to obtain a diffraction pattern of the M-CSF crystal. Finally, a three-dimensional structure of M-CSF is obtained from the diffraction pattern. In most embodiments, the three-dimensional structure includes an M-CSF receptor-binding region.

The present invention is also directed to a method for selecting
15 candidate amino acid substitutions in a protein, based on structural information, and more particularly M-CSF, comprising determining the three-dimensional structure of M-CSF by the methods of the present invention; followed by determining the solvent accessible amino acid residues of the protein, determining which residues are not involved in dimer formation. Applying these criteria, amino acids in M-CSF which
20 are solvent accessible and which are not involved in dimer formation are selected for substitution with non-conservative amino acids. Since M-CSF β has intrachain disulfide bonds involving cysteines 157 and/or 159, we believe the C-terminal region of M-CSF extends from the "rear" of the structure we have solved, providing a variable-length "tether" for membrane-bound forms of M-CSF. Thus, the "front" or
25 receptor-binding region of M-CSF is on the opposite side of the molecules, consisting of solvent-accessible residues in or near helices A, C, and D, including residues from about 6 to 26, 71 to 90, and 110 to 130, respectively, of native M-CSF. Preferred amino acids for substitution and preferred substituting amino acids include but are not
30 limited to: H15 \rightarrow A or L; Q79 \rightarrow A or D; R86 \rightarrow E or D; E115 \rightarrow A; E41 \rightarrow K or R; K93 \rightarrow A or E; D99 \rightarrow K or R; L55 \rightarrow Q or N; S18 \rightarrow A or K; Q20 \rightarrow A or D;

I75 → K or E; V78 → K or R; L85 → E or N; D69 → K or R; N70 → A or E; H9 → A or D; N63 → K or R; and T34 → Q or K. Most preferred are those substitutions give rise to novel M-CSF agonists and M-CSF antagonists. Additionally, the present invention is also directed to a method for producing antagonists and agonists by
5 substituting at least one and preferably fewer than 5 solvent accessible residues per M-CSF monomer.

The invention is also directed to heterodimeric M-CSF in which only one subunit contains substituted solvent accessible amino acids involved in signal transduction and to heterodimeric M-CSF in which each subunit contains different
10 substituted solvent accessible amino acids involved in signal transduction. The present invention is also directed to M-CSF having amino acid substitutions which do not impair binding to the M-CSF receptor. Screening for agonists and antagonists is then accomplished using bioassays and receptor binding assays using methods well known in the art, including those described in the Examples below.

15 In addition the invention is directed to an isolated, purified, soluble and functional M-CSF receptor. The present invention is also directed to a method for screening M-CSF agonists and antagonists using a soluble M-CSF receptor.

A further understanding of the present invention can be obtained by reference to the drawings and discussion of specific embodiments.

20 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a section of a diffraction pattern of an M-CSF α crystal prepared according to the present invention;

Figure 2 is a topology diagram showing the disulfide bonds in truncated dimeric M-CSF;

25 Figure 3 is a stereodiagram of the C-alpha backbone with every tenth residue labelled and with the non-crystallographic symmetry axis indicated by a dotted line;

Figure 4 and 4a present two views of a ribbon diagram highlighting the secondary structural elements of M-CSF. The cysteine residues have been

represented by a ball-and-stick model and the non-crystallographic symmetry axis is indicated by a dotted line;

Figure 5A-C illustrate size-exclusion HPLC analysis of the N Δ 3C Δ 158 M-CSF short clone homodimer (158)(A), N Δ 3C Δ 221 C157S, C159S long clone homodimer (221F)(B), and the short clone/long clone heterodimer (158/221F)(C) and their corresponding biological activities;

Figures 6A-C illustrates size exclusion HPLC(A) and both non-reduced(B) and reduced(C) SDS-PAGE 6 analysis of the preparative purification of M-CSF; and

Figure 7 illustrates the binding of M-CSF and M-CSF muteins to NFS60 cell M-CSF receptors.

DESCRIPTION OF THE PREFERRED EMBODIMENTS DEFINITIONS

As used herein "M-CSF polypeptide" refers to a polypeptide having substantially the same amino acid sequence as the mature M-CSF α , M-CSF β , or M-CSF γ polypeptides described in Kawasaki *et al.*, *Science* **230**:291 (1985), Cerretti *et al.*, *Molecular Immunology*, **25**:761 (1988), or Ladner *et al.*, *EMBO Journal* **6**:2693 (1987), each of which are incorporated herein by reference. Such terminology reflects the understanding that the three mature M-CSFs have different amino acid sequences, as described above.

Certain modifications to the primary sequence of M-CSF can be made by deletion, addition, or alteration of the amino acids encoded by the DNA sequence without destroying the desired structure (e.g., the receptor binding ability of M-CSF) in accordance with well-known recombinant DNA techniques. Further, a skilled artisan will appreciate that individual amino acids may be substituted or modified by oxidation, reduction or other derivitization, and the polypeptide may be cleaved to obtain fragments that retain the active binding site and structural information. Such substitutions and alterations result in polypeptides having an amino acid sequence which falls within the definition of polypeptide "having substantially the same amino acid sequence as the mature M-CSF α , M-CSF β , and M-CSF γ polypeptides."

For purposes of crystallization, preferred lengths of the M-CSF α , β or γ monomers are between about 145 and 180 amino acids (counting from the mature amino terminus), and more preferably between about 145 and 162 amino acids long. A specific monomer that may be present in a crystallizable dimer is M-CSF α and is
5 N'3M-CSF α C'158 (3 amino acids are deleted from the amino terminus and the total length is 155 amino acids). All lengths are inclusive. As used herein the term "M-CSF α (4-158)" denotes an M-CSF having amino acid residues 4 to 158 of the mature, processed M-CSF α polypeptide. Other nomenclature designations for C-terminal and N-terminal truncations of native M-CSF are set forth in U.S. Patent No.
10 4,929,700 which is incorporated herein by reference.

Crystallizable glycosylation variants of the M-CSF polypeptides are included within the scope of this invention. These variants include polypeptides completely lacking in glycosylation and variants having at least one fewer glycosylated site than the mature forms, as well as variants in which the glycosylation
15 pattern has been changed from the native forms. Also included are deglycosylated and unglycosylated amino acid sequence variants, as well as deglycosylated and unglycosylated M-CSF subunits having the mature amino acid sequence (*see*, U.S. Patent No. 5,032,626).

"M-CSF" dimer refers to two M-CSF polypeptide monomers that have
20 dimerized. M-CSF dimers may include two identical polypeptide monomers (homodimers) or two different polypeptide monomers (heterodimers such as an M-CSF α -M-CSF β dimer, an M-CSF long clone and short clone dimer). M-CSF monomers may be converted to M-CSF dimers *in vitro* as described in U.S. Patent No. 4,929,700, which is incorporated herein by reference. Recombinantly expressed
25 M-CSFs may also be variably glycosylated as they exist *in vivo*, partially glycosylated, or completely lacking in glycosylation (unglycosylated). Glycosylated M-CSFs may be produced *in vivo* with carbohydrate chains which may later be enzymatically deglycosylated *in vitro*.

Biologically active M-CSF exhibits a spectrum of activity understood
30 in the art. For instance, M-CSF stimulates the proliferation and function of mature

macrophages via specific receptors on responding cells. Further, M-CSF acts as a mononuclear phagocyte progenitor growth factor. The standard *in vitro* colony stimulating assay of Metcalf, *J. Cell Physiol.* 76:89 (1970) (which is incorporated herein by reference) results primarily in the formation of macrophage colonies when

5 M-CSF is applied to stem cells. Other biological assays are based on M-CSF induced proliferation of M-CSF dependent cells such as the NFS-60 cell line. As used herein "M-CSF having biological activity" refers to M-CSF, including fragments and sequence variants thereof as described herein; that exhibit an art-recognized spectrum of activity with respect to biological systems. Such M-CSF having biological activity

10 will typically have certain structural attributes in common with those of the mature M-CSF forms such as receptor binding site tertiary structure.

Agonists are substances that exhibit greater activity *per se* than the native ligand while antagonists are substances that suppress, inhibit, or interfere with the biological activity of a native ligand. Agonists and antagonists may be produced

15 by the methods of the present invention for use in the treatment of diseases in which M-CSF has been implicated either as a potential treatment (*e.g.*, for treating blood cell deficiencies arising as a side effect of chemotherapy treating fungal infection associated with bone marrow transplants and others) or as having a role in the pathogenesis of the disease (*e.g.*, ovarian cancer, uveitis, atherosclerosis).

20 Crystallization of M-CSF species in accordance with the present invention includes four general steps: expression, purification, crystallization and isolation.

Expression of Recombinant M-CSF

M-CSF crystallization requires an abundant source of M-CSF that may

25 be isolated in a relatively homogeneous form. A variety of expression systems and hosts are suitable for the expression of M-CSF and will be readily apparent to one of skill in the art. Because of the variability of glycosylation and other post-transnational modifications present in M-CSF produced in certain eukaryotic hosts, expression in *E. coli* may provide M-CSF with advantageous properties with

regard to crystallization. Typical *in vitro* M-CSF expression systems are described in U.S. Patent No. 4,929,700, for example.

For use in the present invention, a variety of M-CSF polypeptides can also be readily designed and manufactured utilizing recombinant DNA techniques well known to those skilled in the art. For example, the M-CSF amino acid sequence can vary from the naturally occurring sequence at the primary structure level by amino acid substitutions, insertions, deletions, and the like. These modifications can be used in a number of combinations to produce the final modified polypeptide chain. The present invention is useful for crystallizing such polypeptides and dimers thereof.

In general, modifications of the genes encoding the M-CSF polypeptide are readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (*see*, Gillman and Smith, *Gene* 8:81-97 (1979) and Roberts, S. *et al.*, *Nature* 328:731-734 (1987) and U.S. Patent No. 5,032,676, all of which are incorporated herein by reference). Most modifications are evaluated by screening in a suitable assay for the desired characteristic. For instance, a change in the M-CSF receptor-binding character of the polypeptide can be detected by competitive assays with an appropriate reference polypeptides or by the bioassays described in U.S. Patent No. 4,847,201, which is incorporated herein by reference.

Insertional variants of the present invention are those in which one or more amino acid residues are introduced into a predetermined site in the M-CSF. For instance, insertional variants can be fusions of heterologous proteins or polypeptides to the amino or carboxyl terminus of the subunits. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Nonnatural amino acids (i.e., amino acids not normally found in native proteins), as well as isosteric analogs (amino acid or otherwise) are also suitable for use in this invention. Examples of suitable substitutions are well known in the art, such as the Glu->Asp, Ser->Cys, and Cys->Ser, His->alanine for example. Another class of variants are deletional variants, which are characterized by the removal of one or more amino acid residues from the M-CSF.

Other variants of the present invention may be produced by chemically modifying amino acids of the native protein (*e.g.*, diethylpyrocarbonate treatment which modifies histidine residues). Preferred or chemical modifications which are specific for certain amino acid side chains. Specificity may also be achieved by
5 blocking other side chains with antibodies directed to the side chains to be protected. Chemical modification includes such reactions as oxidation, reduction, amidation, deamidation, or substitution of bulky groups such as polysaccharides or polyethylene glycol (*see e.g.*, U.S. Patent No. 4,179,337 and WO91/21029 both of which are incorporated herein by reference).

10 Exemplary modifications include the modification of lysinyl and amino terminal residues by reaction with succinic or other carboxylic acid anhydrides. Modification with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for modifying amino-containing residues include imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal
15 chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea, 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate, and N-hydroxysuccinamide esters of polyethyleneglycol or other bulky substitutions.

Arginyl residues may be modified by reaction with a number of reagents, including phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and
20 ninhydrin. Modification of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

Tyrosyl residues may also be modified with particular interest in
25 introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues may also be iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

Carboxyl side groups (aspartyl or glutamyl) may be selectively
30 modified by reaction with carbodiimides ($\text{R}-\text{N}=\text{C}=\text{N}-\text{R}^1$), where R and R^1 are

different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4, 4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

5 Conversely, glutaminyl and asparaginyl residues may be deamidated to the corresponding glutamyl and aspartyl residues, respectively, under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the
10 α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

The availability of a DNA sequence encoding M-CSF permits the use
15 of various expression systems to produce the desired polypeptides. Construction of expression vectors and recombinant production from the appropriate DNA sequences are performed by methods well known in the art. These techniques and various other techniques are generally performed according to Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New
20 York (1989), and Kriegler, M., *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, New York (1990), both of which are incorporated herein by reference.

Purification of M-CSF

Purification steps are employed to ensure that the M-CSF is isolated,
25 prior to crystallization, in a relatively homogeneous state. In general, a higher purity solution increases the likelihood of success of subsequent crystallization steps. Typical purification methods include the use of centrifugation, partial fractionation using salt or organic compounds, dialysis, conventional column chromatography (such as ion-exchange, molecular sizing chromatography etc.), high performance liquid

chromatography (HPLC), and gel electrophoresis methods (*see, e.g.,* Deutcher, "Guide to Protein Purification" in *Methods in Enzymology* (1990), Academic Press, Berkely, CA, which is incorporated herein by reference for all purposes). Preferred purification conditions for generating unusually homogeneous M-CSF species as well
5 as purification of these species are disclosed, for example, in U.S. Patent No. 4,929,700 which is incorporated herein by reference. Other purification methods are known and will be apparent to one of skill in the art.

Crystallization of M-CSF

Although many of the same physical principles govern crystallization
10 of polypeptides (including M-CSF dimers) and small molecules, the actual crystallization mechanisms differ significantly. For example, the lattice of small-molecule crystals effectively excludes solvent while that of polypeptide crystals includes substantial numbers of solvent molecules. Thus, special techniques must typically be applied to crystallize polypeptides.

15 Polypeptide crystallization occurs in solutions where the polypeptide concentration exceeds its solubility maximum (i.e., the polypeptide solution is supersaturated). Such "thermodynamically metastable" solutions may be restored to equilibrium by reducing the polypeptide concentration, preferably through precipitation of the polypeptide crystals. Often polypeptides may be induced to
20 crystallize from supersaturated solutions by adding agents that alter the polypeptide surface charges or perturb the interactions between the polypeptide and bulk water to promote associations that lead to crystallization.

Compounds known as "precipitants" are often used to decrease the solubility of the polypeptide in a concentrated solution. Precipitants induce
25 crystallization by forming an energetically unfavorable precipitant depleted layer around the polypeptide molecules. To minimize the relative amount of this depletion layer, the polypeptides form associations and ultimately crystals as explained in Weber, *Advances in Protein Chemistry* 41:1-36 (1991) which was previously incorporated by reference. In addition to precipitants, other materials are sometimes

added to the polypeptide crystallization solution. These include buffers to adjust the pH of the solution (and hence surface charge on the peptide) and salts to reduce the solubility of the polypeptide. Various precipitants are known in the art and include the following: ethanol, 3-ethyl-2,4 pentanediol; and many of the polyglycols, such as polyethylene glycol. A suitable precipitant for crystallizing M-CSF is polyethylene glycol (PEG), which combines some of the characteristics of the salts and other organic precipitants (*see, for example, Ward et al., J. Mol. Biol.* 98:161 [1975] which is incorporated herein by reference for all purposes and McPherson *J. Biol. Chem.* 251:6300 [1976], which was previously incorporated by reference).

Commonly used polypeptide crystallization methods include the following techniques: batch, hanging drop, seed initiation, and dialysis. In each of these methods, it is important to promote continued crystallization after nucleation by maintaining a supersaturated solution. In the batch method, polypeptide is mixed with precipitants to achieve supersaturation, the vessel is sealed and set aside until crystals appear. In the dialysis method, polypeptide is retained in a sealed dialysis membrane which is placed into a solution containing precipitant. Equilibration across the membrane increases the polypeptide and precipitant concentrations thereby causing the polypeptide to reach supersaturation levels.

In the hanging drop technique, an initial polypeptide mixture is created by adding a precipitant to concentrated polypeptide solution. The concentrations of the polypeptide and precipitants are such that in this initial form, the polypeptide does not crystallize. A small drop of this mixture is placed on a glass slide which is inverted and suspended over a reservoir of a second solution. The system is then sealed. Typically the second solution contains a higher concentration of precipitant or other dehydrating agent. The difference in the precipitant concentrations causes the protein solution to have a higher vapor pressure than the solution. Since the system containing the two solutions is sealed, an equilibrium is established, and water from the polypeptide mixture transfers to the second solution. This equilibration increases the polypeptide and precipitant concentration in the polypeptide solution. At the critical concentration of polypeptide and precipitant, a crystal of the

polypeptide will form. The hanging drop method is well known in the art (*see*, McPherson *J. Biol. Chem.* 251:6300 [1976], which was previously incorporated herein by reference).

Another method of crystallization introduces a nucleation site into a concentrated polypeptide solution. Generally, a concentrated polypeptide solution is prepared and a seed crystal of the polypeptide is introduced into this solution. If the concentrations of the polypeptide and any precipitants are correct, the seed crystal will provide a nucleation site around which a larger crystal forms.

In preferred embodiments, the crystals of the present invention will be formed from a dimer of M-CSF polypeptides. Preferred crystals are typically at least about 0.2 x 0.2 x 0.05 mm, more preferably larger than 0.4 x 0.4 x 0.4 mm, and most preferably larger than 0.5 x 0.5 x 0.5 mm. After crystallization, the protein may be separated from the crystallization mixture by standard techniques.

The crystals so produced have a wide range of uses. For example, high quality crystals are suitable for X-ray or neutron diffraction analysis to determine the three-dimensional structure of the M-CSF and, in particular, to assist in the identification of its receptor binding site. Knowledge of the binding site region and solvent-accessible residues available for contact with the M-CSF receptor allows rational design and construction of agonists and antagonist for M-CSFs. Crystallization and structural determination of M-CSF muteins having altered receptor binding ability or bioactivity allows the evaluation of whether such changes are caused by general structural deformation or by side chain alteration at the substitution site.

In addition, crystallization itself can be used as purification method. In some instances, a polypeptide or protein will crystallize from a heterogeneous mixture into crystals. Isolation of such crystals by filtration, centrifugation, etc. followed by redissolving the polypeptide affords a purified solution suitable for use in growing the high-quality crystals necessary for diffraction studies. These high-quality crystals may also be dissolved in water and then formulated to provide an aqueous M-CSF solution having various uses known in the art including pharmaceutical purposes.

Of course, amino acid sequence variants of M-CSF may also be crystallized and used. These mutants can be used for, among other purposes, obtaining structural information useful for directing modification of the binding affinity for M-CSF receptors. As with the naturally occurring forms, the modified
5 M-CSF forms may be useful as pharmaceutical agents for stimulating bone marrow proliferation, overcoming immune suppression and fungal diseases induced by chemotherapy, improving therapeutic efficacy, and lessening the severity or occurrence of side effects during therapeutic use of the present invention. Furthermore, modified M-CSFs may be useful for treatment of disease in which
10 soluble or membrane-bound M-CSF causes or exacerbates the disease state.

Characterization of M-CSF

After purification, crystallization and isolation, the subject crystals may be analyzed by techniques known in the art. Typical analysis yield structural, physical, and mechanistic information about the peptides. As discussed above, X-ray
15 crystallography provides detailed structural information which may be used in conjunction with widely available molecular modeling programs to arrive at the three-dimensional arrangement of atoms in the peptide. Exemplary modeling programs include "Homology" by Biosym (San Diego, California), "Biograf" by BioDesign, "Nemesis" by Oxford Molecular, "SYBYL" and "Composer" by Tripos
20 Associates, "CHARM" by Polygen (Waltham, Massachusetts), "AMBER" by University of California, San Francisco, and "MM2" and "MMP2" by Molecular Design, Ltd.

Peptide modeling can be used to design a variety of agents capable of modifying the activity of the subject peptide. For example, using the
25 three-dimensional structure of the active site, agonists and antagonists having complementary structures can be designed to enhance the therapeutic utility of M-CSF treatment or to block the biological activity of M-CSF. Further, M-CSF structural information is useful for directing design of proteinaceous or non-proteinaceous M-CSF agonists and antagonists, based on knowledge of the contact residues between

the M-CSF ligand and its receptor. Such residues are identified by the M-CSF crystal structure as those which are solvent-accessible, distal to the carboxyl terminal membrane anchoring region not involved in dimer interface stabilizations, and possibly including residues not conserved between human and mouse M-CSF (which
5 does not recognize the human M-CSF receptor).

Example 1

Systematic crystallization trials with M-CSF were made using the hanging drop technique. A microdroplet (5 μ l) of mother liquor is suspended from the underside of a microscope cover slip, which is placed over a well containing 1ml
10 of the precipitating solution. 60-70 initial trials were set up, in which pH, temperature, counterion and precipitant were varied. From these trials, the few that gave promising microcrystals were picked for more careful examination.

It was discovered that suitable crystals may be grown from a 20 μ l drop containing: 10 mg/ml protein, 100 mM MgCl₂, 50 mM Tris.Cl, pH 8.5, and
15 12% PEG 4000. This drop was equilibrated against a reservoir containing 24% PEG 4000. Tiny, needle-like crystals appeared in 2-3 days which were redissolved in 10 μ l water and recrystallized at room temperature. Good quality chunky crystals appeared in 7-9 days in sizes ranging from 0.3 x 0.3 x 0.3 mm to 0.5 x 0.5 x 1.0 mm.

20 Precession photographs revealed the space group to be P2₁2₁2₁ with unit cell dimensions: a=33.53Å, b=65.11Å, c=159.77Å. This gives a unit cell volume of 349084.5Å³, which is consistent with a dimer in the crystallographic asymmetric unit, and 52% of the unit cell volume being occupied by solvent. The crystals diffracted to a resolution of 3Å on a Rigaku rotating anode X-ray generator
25 (Danvers, Massachusetts) operated at 50 kV and 60mA, and to 2.6Å in synchrotron radiation.

Screening for heavy atom derivatives was done by soaking crystals into solutions of heavy-metal salts. Zero-level precession pictures of the soaks were used

to identify potential derivatives. About 30 different soaking conditions were examined, of which 4 potential derivatives were identified. Unfortunately, some soaks caused the crystals to exhibit non-isomorphism (i.e., the heavy atom soaks induced a change in cell dimensions, making them unusable for phase calculation).

5 Three-dimensional intensity data were collected on film using an oscillation camera on the X-ray beam-line at the National Synchrotron Light Source, Brookhaven. Several other data sets, of native (underivatized M-CSF) as well as potential derivative crystals have been collected on a Rigaku X-ray generator. The following data sets were collected.

10

Crystal	Resolution(Å)	N(observations)	N(unique)	X-Ray Source
Native	2.8	27922	7311	Synchrotron
Native	2.9	35236	7002	Rigaku (film)
Native	3.5	5144	5116	Rigaku (diffractometer)
15 K ₂ Hg(SCN) ₄	3.5	15885	4119	Rigaku (film)
UO ₂ Cl ₂	3.5	25492	5048	Rigaku (film)
Cis-Pd(NH ₃) ₂ Cl ₂	3.1	26122	6304	Synchrotron

Example 2

20 Recombinant M-CSF polypeptides were purified from *E. coli*. and renatured to form a disulfide-linked dimeric protein as described in U.S. Patent No. 4,929,700. Crystallization of the resulting unglycosylated M-CSF α protein (amino acids 4-158 in homodimeric form) was performed by the hanging drop method. Glass microscope plates were siliconized prior to use by dipping immersion into a 1% (volume:volume) solution of the organosilane compound, Prosil-28 (PCR

Incorporated, Gainesville, Florida, 32602) washing the treated glass plates with water, and baking at 180 degrees.

5 A 2 mg/ml aqueous solution of purified human recombinant M-CSF was dialyzed and concentrated against 50 mM Tris-HCl (pH 8.5) using a dialysis tubing having a 10kD cutoff. The final concentration of polypeptide (10 mg/ml) was determined by ultraviolet spectrophotometry at 280 nm.

About 7 microliters of the concentrated solution was mixed in each well of the spot plate with 7 microliter of 20% (v/v) PEG 4000, 0.2 M $MgCl_2$, 0.1 M Tris-HCl (pH 8.5). The spot plate was then placed in a clear plastic sandwich box containing 20 ml of 23% PEG 4000, 0.2 M $MgCl_2$, 0.1 M Tris-HCl (pH 8.5) and the box was immediately sealed and stored at room temperature. Minor variations in this procedure such as altering buffer conditions are within the scope of the present invention. For example, in a preferred embodiment of the present invention, buffer conditions were altered to include 150 mM $MgCl_2$ and 24% PEG 4000.

15 After 3-5 days, small microcrystals having a size of 0.1 x 0.1 x 0.05 mm appeared in each well. These microcrystals were isolated and redissolved in 25 microliter of 50 mM Tris-HCl and allowed to stand at room temperature. The purified M-CSF crystallized from solution into large hexagonal prism shaped crystals ranging in size from 0.3 x 0.3 x 0.3 mm to 1 mm x 2 mm x 0.5 mm. These crystals were stable at room temperature for at least three months. In some instances, an artificial mother liquor was prepared using 23% PEG 4000 and 150 mM $MgCl_2$ crystals were then added to this mother liquor. In these cases, the crystals were removed from the mother liquor immediately prior to analysis.

25 Using reducing and non-reducing SDS-PAGE analysis, the M-CSF in the crystals was shown to be identical in molecular weight to the biologically active starting material. Thus, the M-CSF structure obtained from the crystals is likely to be essentially identical to the structure of biologically active M-CSF.

Example 3

Glass microscope slides were prepared as described in Example 2. 7 microliters of the same concentrated M-CSF α protein solution was mixed in each well of the spot plate with 7 microliter of 30% (v/v) PEG 4000, 0.2 M ammonium acetate, 0.1 M acetate buffer (pH 7.5). The spot plate was then placed in a clear plastic sandwich box containing 20 ml of 30% PEG 4000, 0.2 M ammonium acetate, 0.1 M acetate buffer (pH 7.5) and the box was immediately sealed and stored at room temperature. After 3-5 days, thin, plate-like, fragile crystals having a size of approximately 0.3 x 0.3 x 0.05 mm appeared.

10

Example 4

Preliminary X-ray Analysis

X-ray crystallographic analysis using precession photographs showed that the crystals produced in Example 2 have an orthorhombic crystal lattice in the P2₁2₁2₁ space group with cell dimensions a=33.54, b=65.26, c=159.63 d = 90.0, c = 90.0 and f = 90.0 angstroms and diffract to a nominal resolution of 2.6 angstroms using synchrotron radiation. These data provided a unit cell volume of 348084.5 angstroms³, which is consistent with a dimer in the crystallographic asymmetric unit with 52% of the unit cell volume being occupied by solvent. Fig. 1 is a 12-degree precession photograph of the Okl-zone section of the M-CSF crystal. The photograph was taken using a precession camera manufactured by Enraf-Nonius Company (Delft, Holland), mounted on a Rigaku RU-200 X-ray generator operated at 50kV and 50mA.

20

Example 5
Testing of M-CSF Receptor Binding Ability
Using Soluble M-CSF Receptor

An essential step in the biological function of M-CSF *in vivo* is the binding to the M-CSF receptor, also referred to as the *c-fms* gene product. Recombinant human soluble M-CSF receptor (rhsM-CSFR), representing amino acids 20 to 511 (Coussens, L *et al.*, *Nature*, 320:277 (1986)) was used as an *in vitro* assay reagent to test the receptor-binding ability of M-CSF proteins. To generate a soluble form of the transmembrane receptor, only the extracellular domain of the human M-CSF receptor was expressed in a baculovirus/insect cell recombinant expression system. In order to purify the soluble receptor without adversely effecting tertiary or quaternary structure, non-denaturing chromatographic methods were chosen, as described below. Other choices exist for the purification of the recombinant receptor. Affinity chromatography may be employed when either a suitable antibody to or ligand for the receptor are available. Alternatively, "tags" may be added to the C-terminus of the recombinant receptor, *i.e.*, KT3 antibody recognition sequence, and purified by an anti-tag antibody, *i.e.*, KT3, column, for use in affinity chromatography. In expression systems in which the rhsM-CSFR is glycosylated, lectin chromatography can be used to enrich for specific glycoproteins.

The rhsM-CSFR can be used to study ligand/receptor interactions as well as ligand-induced receptor dimerization. The assay used to detect ligand/receptor binding employed the use of size exclusion-HPLC, essentially as described in European Patent Application WO92/21029, C. Cunningham, *et al.*, with the following modifications: the column used was a Superose 6 (Pharmacia LKB Biotechnology, Inc.) and the mobile phase was PBS at 0.5 ml/min and a M-CSF to rhsM-CSFR ratio of 1:2. At this ratio, the M-CSF/rhsM-CSFR complex chromatographed with an apparent hydrodynamic radius of 190,00 molecular weight, the molecular weight expected for a M-CSF(rhsM-CSFR)₂ complex. Other assays may be employed to measure ligand/receptor binding or receptor dimerization such

as chemical crosslinking and SDS-PAGE or immunoprecipitation and SDS-PAGE. Molecules that inhibit receptor dimerization but not ligand binding provide another method to antagonize M-CSF actions.

The DNA encoding rhsM-CSFR was cloned for expression in insect
5 cells using the following general strategy. The portion of the *c-fms* gene corresponding to amino acids one to 511 was amplified from human macrophage cDNA by polymerase chain reaction (PCR) using an upstream primer of:
5'-GCGTACCATGGGCCAGGAGTTCTGC-3' (SEQ ID NO. 9) and a downstream primer of:
5'-AGTCGAGGATCCTCAATCCGGGGGATGCGTGTG-3' (SEQ ID NO. 10). The underlined
10 sequences are the NcoI and BamHI restriction sites used to subclone the PCR product into the pAcC5 vector (Luckov *et al.*, *Bio/Technology* 6:47-55). The pAcC5:hsM-CSFR vector was expressed in SF9 insect cells using a baculovirus helper vector as previously described (Summers, *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures* (1987)).

15 Approximately two liters of serum-free 72-hour conditioned medium was collected by centrifugation and filtration from SF9 cells infected with pAcC5:hsM-CSFR construct described above. The material was diafiltered with DEAE buffer A [10 mM Tris, pH 8.8, containing the following protease inhibitors (which were added all buffers throughout the purification): 1 mM EDTA, 2 µg/ml
20 leupeptin and 100 µM PMSF] and concentrated 20-fold with a 20,000 molecular weight cut-off Pyrostat Ultrafiltration Membrane (Sartorius). The retentate was loaded onto a DEAE Sepharose column (Pharmacia LKB Biotechnology, Inc., Piscataway, New Jersey) having a bed volume of 100 ml that had been pre-equilibrated with DEAE Buffer A. Elution was at 5 ml/min with a 0-0.8 M NaCl
25 gradient in 500 ml of DEAE Buffer A. Fractions enriched in rhsM-CSFR were detected Western Analysis [Burnett, R., *Anal. Biochem.*, 112:195 (1981)] and dot blot analysis of serially diluted fractions, using anti-c-fms monoclonal antibodies (Oncogene Sciences, Inc.). The dot blot assay was used throughout the purification to identify fractions containing rhsM-CSF. Enriched fractions were pooled, made 0.8
30 M in ammonium sulfate, adjusted to pH 7.0 and loaded onto a Phenyl TSK-5-PW

HPLC column (7.5 x 75 mm) (BioRad). The column was eluted at 1 ml/min with a decreasing ammonium sulfate gradient over 45 minutes, peak fractions were pooled and concentrated 10-fold with a stir cell concentrator using a YM30 membrane (Amicon). The retentate was chromatographed with FG3000XL size exclusion
5 column (DU PONT, Wilmington, Delaware) using a mobile phase a phosphate-buffered saline (PBS) at 3 ml/min. The purified receptor was pooled, concentrated to 1 mg/ml as above and stored at 4°C. This process recovered 650 µg of rhsM-CSFR, purified 200-fold. The preparation was about 95% homogeneous as assayed by SDS-PAGE stained with Coomassie Blue.

10

Example 6

Crystallization of M-CSF/Soluble M-CSF Receptor Complex

To crystallize the M-CSF/rhsM-CSFR complexes, glass microscope slides are prepared as described in Example 2. The M-CSF composition used is incubated with a purified soluble form of the M-CSF receptor, truncated at a residue
15 before the transmembrane region, to form an M-CSF/ receptor complex. In certain cases, the rhsM-CSFR is deglycosylated prior to the size exclusion step by incubation with N-glycanase (Genzyme, Cambridge Massachusetts) according to the manufacturer's instructions. A small quantity of M-CSF/receptor solution is mixed in each well of the spot plate with a comparable quantity of a drop solution (such as
20 about 20% (v/v) PEG 4000, 0.2 M MgCl₂, 0.1 M Tris-HCl (pH 8.5)). The spot plate is then placed in a clear plastic sandwich box containing a small amount of precipitant solution (such as about 23% PEG 4000, 0.2 M MgCl₂, 0.1 M Tris-HCl (pH 8.5)). The box is immediately sealed and stored at room temperature.

After a few days, crystalline M-CSF-receptor complex is isolated and
25 redissolved in a solution containing about 50 mM Tris-HCl and is allowed to stand at room temperature. The purified M-CSF-receptor complex crystallizes from solution to form crystals for X-ray structural analysis. To facilitate solution of the crystal structure of such complexes, truncated, non-glycosylated forms of the rhsM-

CSFR (described above) which retain M-CSF binding ability may be employed to generate M-CSF-receptor complex crystals.

Example 7

The biological activity of the non-glycosylated, truncated sequence used
5 in Examples 2 and 3 was shown to be equal to that of the mature protein purified from human urine (Halenbeck, R., *et al.*, *Bio/Technology*, 7:710-715 [1989]). As noted, the resulting crystals had an orthorhombic crystal lattice in the $P2_12_12_1$ space group, with cell dimensions $a=33.54$, $b=65.26$, and $c=159.63$ Å. Intensity data were collected using imaging plates mounted on a Weissenberg camera modified for
10 macromolecular crystallography at the Photon Factory in Tsukuba, Japan. Native data to a nominal resolution of 2.0\AA , and mercury and platinum derivative data were collected using 1.0\AA radiation. Two crystal settings were used to collect native data [$R_{\text{merge}}(I)=7.0\%$, using all measurements with $I>0.0$].

Heavy atom derivatives of M-CSF crystals were prepared by soaking
15 crystals in heavy atom compounds dissolved in the reservoir solution. Isomorphous and anomalous difference Patterson maps clearly revealed one site for the mercury and two sites for the platinum derivative. Anomalous and isomorphous phase information as used in initial phase refinement with the PROTEIN program package. The final figure of merit was 0.62 ($8.0\text{-}3.0\text{\AA}$, 6960 reflections). After solvent
20 flattening, B.C. Wang, *Methods Enzymol.*, 115:90 (1985), two bundles of four alpha helices related by an approximate two-fold axis could be seen in the electron density map. Rotation and translation parameters of this non-crystallographic axis were refined by a density correlation method, J.M. Cox, *J. Mol. Biol.* 28:151 (1967). Phases were then iteratively refined by molecular averaging and solvent flattening,
25 G. Bricogne, *Acta Cryst.*, 32:832 (1976), using an envelope calculated by putting 5\AA spheres around all the atoms in the four helical bundle. Chain tracing and model building were done in the resulting map, (using the program FRODO), T.A. Jones, *Methods Enzymol.* 115:157 (1985), keeping the original MIR map as a reference.

The starting partial model for refinement contained only a polyalanine backbone for eight helices making up the two bundles. Positional refinement using the program XPLOR, A.T. Brunger, *J. Mol. Biol.* **203**:803 (1985), gave an R-factor of 0.49 to 3.0Å. Phase combination with the refined MIR phases resulted in a map of sufficient quality to allow the tracing of two long loops traversing the four helical bundle and a short loop connecting two of the helices. Two strong peaks in the density, one at the top of the first helix, and the second lying directly on the molecular two fold axis, were assigned as disulfide bonded cysteines. The number of residues between these two peaks uniquely identified the position in the sequence of these cysteines and consequently the sequence of the intervening residues. This initial registration was confirmed by the presence of a number of regions of strong density corresponding to aromatic side chains in the sequence. Partial model phase combination using the added loops and those side chains that were visible allowed the remaining residues to be registered, thus determining the overall topology of the molecule. The presence of seven disulfide bonds in the dimer served as important "tether points" to confirm the correctness of the tracing.

As shown in Fig. 2, the overall topology of this form of M-CSF is that of an antiparallel four α -helical bundle, in which the helices run up-up-down-down, unlike the more commonly observed up-down-up-down connectivity of most four helical bundles. A long crossover connection links helix A to helix B and a similar connection is found between helices C and D.

A striking difference from other cytokines and other four helix bundle structures is that the truncated M-CSF α forms a disulfide-linked dimer, in which the bundles are linked end-to-end, forming an extremely flat, elongated structure (approximate dimensions 85 x 35 x 25 Å) as shown in Figs. 3 and 4. There are three intramolecular disulfide bonds in each monomer (Cys7-Cys90, Cys48-Cys139, Cys102-Cys146) all of which are at the distal end of the molecule. One interchain disulfide bond (Cys31-Cys31) is located at the dimer interface with the noncrystallographic two-fold symmetry axis passing through it as shown in Figs. 3 and 4. Mutation experiments indicate that all of the cysteine residues in this form of

M-CSF may be necessary for full biological activity. The structure described herein suggests that their role is primarily structural rather than being related to receptor recognition.

Appendix 1 provides the three-dimensional structure of the truncated recombinant M-CSF α dimer as identified by the alpha-carbon positions of the amino acid residues in the sequence. The five carboxy terminal amino acids of each polypeptide of the dimer were not included. As will be recognized to those of skill in the art, the information in Appendix 1 is provided in the format used by the Brookhaven Protein Data Bank.

As shown, the molecule has an unusual topology which identifies important regions of M-CSF with regard to M-CSF receptor binding. Specific residues in helices A, C, and D appear to be involved in the specificity of the interaction. Altering solvent accessible residues in these regions by site directed mutagenesis to increase or decrease side-chain interactions with the receptor may be useful to generate M-CSF agonists or antagonists. For example, changing one or more histidines to non-hydrogen-donor amino acids of similar size may create an M-CSF with altered receptor binding ability.

Example 8

Preparation of M-CSF Heterodimers

Purification of M-CSF Monomers:

E. coli harboring the pL-M-CSF vector for N Δ 3C Δ 158M-CSF α described in U.S. Patent No. 4,929,700 or N Δ 3C Δ 221 M-CSF β C157S, C159S (Kawasaki, *et al.*, in *Colony-Stimulating Factors*, Dexter, T., Garland, J., and Testa, N., eds. [1990]) were grown in 1 liter of minimal salts medium containing glucose and the appropriate antibiotic. Expression of M-CSF was induced by shifting the temperature to 39°C for 4 hr. following the addition of casamino acids to 2%. The cells were harvested by centrifugation and lysed by sonication in 50 mM Tris (pH 8.5), 10 mM EDTA. The cell debris was recovered by centrifugation, washed with

30% sucrose containing 10 mM EDTA, and a portion of the refractile body paste was solubilized in 8 M urea under reducing conditions. After incubation at 37°C for 30 min., the solubilized M-CSF was clarified by centrifugation and filtration, and then loaded onto a Bio-Gel TSK-DEAE-5-PW column (7.5 x 75 mm) (BioRad Laboratories, Richmond, California) equilibrated in 8 M urea in 10 mM Tris (pH 8.5), 5 mM DTT, 0.5 mM EDTA. The monomeric M-CSF was eluted with a 45-min, 0-0.6 M NaCl gradient. The M-CSF peak fractions were pooled and concentrated to 10 mg/ml with a Centricon 10 microconcentrator (Amicon).

Formation and Analysis of Active M-CSF Heterodimers

The M-CSF homodimers were refolded by diluting to a protein concentration of 0.5 mg/ml in precooled 50 mM Tris (pH 8.5), 5 mM EDTA, 2 mM reduced glutathione and 1 mM oxidized glutathione, and then incubating at 4°C. The heterodimer was refolded by diluting 158 and 221F monomer pools to 1 mg/ml in the same buffer. To monitor the refolding, size exclusion high pressure liquid chromatography (SE-HPLC) analysis was performed by immediately injecting reaction samples onto a G3000SW Ultropack (Pharmacia LKB Biotechnology, Inc., Piscataway, New Jersey) column size-exclusion (7.5 x 600 mm) equilibrated in PBS (pH 6.8).

Fractionated products were analyzed on reducing SDS-PAGE and stained with Coomassie, according to the method of Laemmli, *Nature* (Canada) 227:680-685 (1970). Biological activity was determined using the M-CSF dependent NFS-60 bioassay (See Example 10 below). Antibody neutralization experiments were carried out by pre-incubating approximately 5,000 units of M-CSF dimer with varying dilutions of the neutralizing M-CSF 5H410 Mab (made to refolded *E. coli* CΔ 150 M-CSFα dimer) prior to bioassay. (Halenbeck *et al*, *Bio/Technology* 7:710 (1989)

The heterodimeric M-CSF product was designed to consist of one chain of short clone (from amino acid 4 to 158) and one chain of long clone (from amino acid 4 to 221). The long-clone chain (221F) also contained substitutions of serine for

the two non-essential cysteines (at 157 and 159) to minimize the possibility of higher-order oligomer formation.

Solubilized refractile bodies of M-CSF 158 and 221F were separately chromatographed by DEAE-HPLC in 8 M urea. Only one major protein peak eluted in each case, and the peak fractions were pooled, based on an analysis of purity by non-reducing SDS-PAGE and Coomassie staining (data not shown). The resulting monomer was over 90% pure in each case. The monomers were separately concentrated to 10 mg/ml, diluted in refolding buffer, and refolded at 4°C.

To compare the rates of dimerization of short- and long-clone M-CSF, 20 µl of each refolding reaction was injected on a SE-HPLC column at 0, 2, 18 and 72 hr. The amount of dimeric M-CSF formed was determined from the peak area at the molecular weight expected for dimer. In both refolding reactions the M-CSF was mostly equilibrated to monomer at t=0 and had become about 40% dimeric by 2 hr and nearly 75% dimeric by 18 hr. The similarity of the ratio of dimer to monomer between the refolded 158 and 221F strongly suggests that the rate of dimer formation is the same for long- and short-clone M-CSF. Thus, when equal moles of 158 and 221F are present in a refolding reaction, the final relative ratios of 158 homodimer to 221F homodimer to 158/221F heterodimer are predicted to be 1:1:2 (Similar distributions have been observed *in vivo* for isozymes of lactate dehydrogenase.)

Biological Activity of Refolded Homodimers and Heterodimers

The biological activity of the refolded homodimers and heterodimers described above, was examined using the *in vitro* M-CSF-dependent NFS-60 bioassay (See Example 10 below). Figures 5A-C show the result of these studies. These SE-HPLC and biological activity profiles analyzed after 72 hr of refolding, show that the heterodimer, Figure 5C, displays activity very similar to that of the two homodimers, (Figures 5A and B). Given that the separation of the heterodimer from the homodimers was nearly complete, it can be concluded that the heterodimer is fully biologically active *in vitro*.

To verify that the M-CSF protein eluting from these columns at the predicted heterodimeric position (between the two homodimers) actually did consist of equal moles of short- and long-clone monomers, analysis of a preparative purification of the 158/221F heterodimer was carried out. Phenyl-HPLC was performed as described above and was shown to completely resolve the heterodimer from the 158 and 221F homodimers, as seen in Figure 6A.

Preparative Purification of M-CSF Heterodimers:

The refolded M-CSF was adjusted to pH 7.0 with 1 N HCl, and ammonium sulfate was added to 1.2 M. The protein was loaded onto a Bio-Gel TSK-Phenyl-5-PW column (7.5 x 75 mm) (BioRad, Richmond, California) equilibrated in 1.2 M ammonium sulfate, 100 mM phosphate (pH 7.0). The M-CSF was eluted with a decreasing gradient of ammonium sulfate from 40% to 80% buffer B (10 mM phosphate, pH 7.0) in 24 min.

Reducing and non-reducing SDS-PAGE (Figures 6B and C) showed that internal controls (the 158 and 221F dimers) were purified to approximately 95% homogeneity by this column, and each consisted of the single expected monomeric band. The gel analysis also showed that the heterodimer was purified to approximately 95% homogeneity and that it consisted of equivalent amounts of 158 and 221F monomers. Recovery of purified 158/221F heterodimer from refractile body paste to final product, was greater than 15%.

The bioactivity of the dimeric M-CSF species was determined and, when compared to the A_{280} profile in Figure 6A, confirms the finding that the heterodimer is fully active. The specific activity of the 158/221F heterodimer, calculated using the peak fraction, was 8.0×10^7 units/mg, compared to 9.0×10^7 and 6.8×10^7 units/mg for 158 and 221F homodimers, respectively.

The biological activity of all three dimer species was neutralized to the same extent in serial dilution neutralization experiments using the 5H410 M-CSF Mab in the NFS-60 bioassay. This antibody also neutralizes "naturally refolded" Chinese hamster ovary cell (CHO)-expressed M-CSF in a similar fashion. This result further

suggests that the refolded conformation of the new M-CSF heterodimer is essentially native-like, at least with regard to the region within the first 150 amino acids that is responsible for *in vitro* activity.

Example 9

5 Selection of Amino Acid Substitutions in M-CSF Based on Crystallographic Data

The X-ray crystallographic data described above provided sufficient structural information regarding M-CSF to be able to identify a limited subset of the amino acids in the protein that are likely to be crucial for M-CSF receptor binding and biological activity and thus which represented likely candidates for mutagenesis with the ultimate goal of providing M-CSF muteins having altered biological activity (i.e., agonists or antagonists). Based on this information, several criteria were used to generate a list of possible target amino acids for substitution.

The first criterion was solvent exposure or solvent accessibility, which refers to amino acids residues at the surface of the protein. Residues having a solvent accessible surface area of greater than about 0.25 and preferably greater than about 0.4 are preferred based on normalization of the surface area of the amino acid accessible when in the tryptptide gly-x-gly (Kabsch, W. *et al.*, *Biopolymers* 22:2577 (1983)). Residues were chosen which do not interact with other parts of the protein such as the dimer interface in order to maintain the relative orientation of monomers and to avoid disturbing the process of protein folding. Still another criterion used in certain instances in selecting candidate amino acid substances is the relationship of the residues to corresponding residues in mouse M-CSF. Another important selection criterion was that the substitutions be non-conservative so as to attempt to disrupt possible hydrogen bonding or hydrophobic interactions with M-CSF receptor residues.

Table 1 lists exemplary amino acid residues and exemplary substitutions. Using the criteria for selecting candidates for substitutions set forth

above, those of ordinary skill in the art may readily ascertain other possible candidates for substitution.

Table I
Candidate Substitutions

<u>5</u>	<u>Wild Type Amino Acid and Location</u>	<u>Substitutions</u>
	His (H) 15	Ala(A) or Leu(L)
	Gln (Q) 17	Ala(A) or Glu(E)
	Gln (Q) 79	Ala(A) or Asp(D)
	Arg (R) 86	Glu(E) or Asp(D)
10	Glu (E) 115	Ala(A)
	Glu (E) 41	Lys(K) or Arg(R)
	Lys (K) 93	Ala(A) or Glu(E)
	Asp (D) 99	Lys(K) or Arg(R)
	Leu (L) 55	Gln(Q) or Asp(N)
15	Ser (S) 18	Ala(A) or Lys(K)
	Gln (Q) 20	Ala(A) or Asp(D)
	Arg (R) 21	Ala(A), Glu(E), or Asp(D)
	Ile (I) 75	Lys(K) or Glu(E)
	Val (V) 78	Lys(K) or Arg(R)
20	Leu (L) 85	Glu(E) or Asn(N)
	Asp (D) 69	Lys(K) or Arg(R)
	Asn (N) 70	Ala(A) or Glu(E)
	His (H) 9	Ala(A) or Asp(D)
	Asn (N) 63	Lys(K) or Arg(R)
25	Thr (T) 34	Gln(Q) or Lys(K)

It is not expected that every candidate substitution listed will result in the production of M-CSF agonists or antagonists (see Example 12 below). Rather they represent a non-exclusive list of candidates likely to result in the production of agonists or antagonists based on the selection criteria set forth above. It should also

be noted that even if a variant does not act as an agonist or antagonist when compared with native M-CSF, the variant is still useful for conventional uses of the ligand (if it retains the same activity as the ligand) or as for example, a diagnostic reagent.

Example 10

5 Preparation of H9A, H15A M-CSF Muteins

A variety of M-CSF muteins with altered solvent-accessible residues from regions of the M-CSF mature N terminus and helices A, C, and D were constructed using techniques known in the art. For example, two histidines in the N-terminal/A helix region were changed to alanine through site-directed mutagenesis of
10 a truncated form of M-CSF α (encoded by pLCSF158A). Involvement of one of three M-CSF histidine residues in M-CSF receptor interaction was implicated by our observation that diethylpyrocarbonate (DEPC) modification of histidines in M-CSF at a 1:100 DEPC:histidine ratio (as described in *Meth. in Enzymol.* 47:431 (1977)) significantly reduced bioactivity.

15 Plasmid DNA pLCSF158A was prepared from the *E. coli* strain HW22 carrying the plasmid pLCSF158A (U.S. Patent No. 4,929,700, Example 6, "*E. coli* strain HW22 transformed with pJN653 containing the asp₃₉SCSF/N Δ 3C Δ 158 gene"). The strain was grown in 350 ml R2 media (2X Luria Broth containing 1% sodium chloride and no glucose, *J. Bact.*, 74:461 (1957)) containing 50 micrograms/ml
20 ampicillin at 30° C with shaking overnight. Plasmid DNA was prepared from the cells using a Qiagen-tip 100 column according to the manufacturer's directions.

Twenty micrograms of pLCSF158A DNA were digested with 66 units of *Hind*III and 66 units of *Stu*I at 37°C for 3hr. 20 min. in 200 microliters 1X New England Biolabs NEBuffer 2 (New England Biolabs, Beverly, Massachusetts). The
25 DNA was extracted with phenol and chloroform, then ethanol precipitated. The DNA was treated with one unit of Calf Intestinal Alkaline Phosphatase in 100 microliters of 1X Dephosphorylation Buffer, supplied by Boehringer Mannheim (Indianapolis,

Indiana), at 37°C for 30 min. An additional unit of Calf Intestinal Alkaline Phosphatase was added to the reaction and incubation was continued at 50°C for 1 hr.

The resulting DNA was then run on a 1% FMC Bioproducts (Rockland, Maine) Sea KEM® GTG® agarose gel. The 5.7 kb pLCSF158A fragment
5 was cut from the gel and purified on Qiagen (Chatsworth, California) Qiaex beads according to the manufacturer's directions.

Polymerase chain reaction (PCR) was then performed and a PCR product was produced that contained a mutagenized M-CSF sequence in which histidines 9 and 15 (counting from the mature N-terminus) were altered to alanine
10 (generating an H9A, H15A PCR fragment). The 5' portion of the M-CSF gene was amplified from the plasmid pLCSF158A in a PCR reaction using the primers LF73 and LF74. Details of PCR are provided by Mullis, K. *et al.*, U.S. Patent No. 4,683,202; Ehrlich, H., U.S. Patent No. 4,582,788; Saiki *et al.*, U.S. Patent No. 4,683,195; Mullis, K.B., *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273 (1986);
15 Saiki *et al.*, *Bio/Technology* 3:1008-10012 (1985); and Mullis, K.B. *et al.*, *Meth. Enzymol* 155:335-350 (1987), all of which are incorporated herein by reference. The sequences of these primers are:

LF73:

5'-AGGTGTCTCATAGAAAGTTCGGACGCAGGCCTTGTCATGCTCTTCATAATCCTTGG-3'
20 (SEQ ID NO. 1)

LF74:

5'-CAGGAGAAAGCTTATGTCTGAATATTGTAGCGCCATGATTGGGAGTGGAGCCCTGCAG-3'
(SEQ ID NO. 2)

The expected PCR product was designed to include 337 bp of
25 pLCSF158A sequence, with the *Hind*III and *Stu*I sites located at each end of the product for cloning, and the histidine codons for His 9 and His 15, CAC, mutated to an alanine codons, GCC.

This product was amplified in four separate PCR reactions each containing 100 ng of pLCSF158A DNA, 50 pmoles LF73, 50 pmoles LF74, 37.5 µM

dNTPS, 5% glycerol, 1X Perkin Elmer Cetus PCR Buffer, and 2.5 units of Perkin Elmer Cetus AmpliTaq® DNA Polymerase in a 100 microliter volume. The amplification was carried out in a Perkin Elmer Cetus DNA thermocycler. Before adding the AmpliTaq®, the reactions were brought to 95°C. The amplification was
5 carried out for 25 cycles ramping to a denaturation temperature of 95°C in 1 sec., denaturing at 95°C for 1 min.; ramping to an annealing temperature of 68°C in 1 sec., annealing at 68°C 1 min.; ramping to an extension temperature of 72°C in 30 sec., extending at 72°C 1 min., 30 sec. Final extension was carried out at 72°C for 10 min.

10 Five microliters of each reaction were run on a 3% agarose gel (1.5% FMC Bioproducts SeaKem® GTG® agarose, 1.5% FMC Bioproducts NuSeive® GTG® agarose in Tris-Borate buffer) (FMC Bioproducts, Rockland, Maine). Gels were then stained with ethidium bromide. For each reaction, a major band was visible at approximately 337 bp.

15 The four reactions were pooled, extracted with phenol and chloroform, precipitated with ethanol, resuspended and digested with 250 units of *StuI* in a final volume of 500 microliters 1X NEBuffer 2 at 37°C for 2hr., 500 units of *HindIII* were added to the reaction, the volume increased to 1 ml in 1X NEBuffer 2 and digestion was continued at 37°C for an additional 2.5hr. The DNA was electrophoresed on a
20 3% agarose gel. The 300 bp digested product was cut from the gel and purified on Qiagen Qiaex beads according to the manufacturer's directions.

Approximately 68 ng of the *HindIII/StuI* digested PCR product was then ligated to approximately 28 ng of the 5.7 kb *HindIII/StuI* digested pLCSF158A vector DNA at an insert-to-vector ratio of approximately 5:1. Ligation was carried
25 out with 1 unit of Boehringer Mannheim T4 DNA ligase in 1X ligation buffer, supplied by the manufacturer, in a 20-microliter volume at 16°C overnight. As a control 28 ng of the 5.7 kb *HindIII/StuI* digested pLCSF158A vector DNA was ligated to itself under the same conditions with no insert present.

Half of each ligation mixture was used to transform competent *E. coli*
30 DG116 (ATCC# 53606) cells using a protocol similar to the calcium chloride

procedure described in Molecular Cloning a Laboratory Manual Maniatis *et al.*, Cold Spring Harbor Laboratory (1982). Transformed cells were allowed to express at 30°C with no selection for 90 min., plated on R2-4 (10g tryptone, 5g yeast extract, 5g NaCl, 2 drops antifoam A, 4 ml 50% glucose and 15g agar in 1 liter) plates
5 containing 50 micrograms/ml ampicillin. The plates were incubated at room temperature 72 hr. One fourth of each transformation was plated. For the ligation containing the insert, 66 ampicillin resistant colonies appeared on the plates. For the ligation with no insert, no colonies appeared.

One of these colonies, designated strain TAF172-2, was picked and
10 cultured in 350 ml R2 broth with 50 micrograms/ml ampicillin at 30°C with shaking overnight. A frozen stock in 40% glycerol was made from this culture and stored at -70°C. DNA was isolated from the culture using Qiagen-tip 100 columns as described above.

The purified DNA, pTAF172-2, was sequenced using the di-deoxy
15 method and shown to contain the sequence of pLCSF158A coding for M-CSF N Δ 3C Δ 158 with His 9 and His 15 mutated to encode Ala.

The M-CSF mutein N Δ 3C Δ 158 H9A, H15A encoded by pTAF172-2 was expressed, purified, refolded to form dimeric protein and assayed essentially as described in U.S. Patent No. 4,929,700 Example 5, using 8M urea as a denaturant
20 and in the DEAE purification step.

The N-terminal sequence of the purified mutein was determined through 20 cycles, using a standard automated Edman degradation method, and shown to be identical to that of the parental N Δ 3C Δ 158 M-CSF α reference protein except that His 9 and His 15 had been altered to Ala. Protein concentration was determined
25 using A280 and an extinction co-efficient of 0.68.

The purified mutein dimers were subjected to bioassay using NFS-60 cells which is an M-CSF dependent cell line which forms colonies in the presence of active M-CSF. Standards and purified mutein samples were serially diluted 1:2 in RPMI media (10% fetal bovine serum) in a 50 microliter volume in 96-well
30 microtiter plates. 50 microliters NFS-60 cells (ATCC NO. CRL 1838), maintained

in 4000U/ml M-CSF, washed 2X, and diluted to a concentration of 1×10^5 cell/ml, were added to each sample well. Plates were incubated at 37° C, 5% CO₂ for 44h. 5 mg/ml MTT dye was added to each sample and incubation continued for 4h. 20% SDS was added to each sample. The plates were wrapped in foil and left overnight.

- 5 The absorbance of each sample was read at 570 nm and compared to a standard of known M-CSF concentration. The H9A, H15A mutein showed a specific activity of 7.6×10^3 U/mg compared to 6.9×10^7 U/mg for the parental M-CSF N Δ 3C Δ 158 reference in the same assay. This represents a nearly 10,000 fold reduction in biological activity for the mutein. The same M-CSF mutein preparation was shown
- 10 to have greatly decreased M-CSF receptor-binding ability using the NFS-60 receptor competition assay described in Example 9. Because the H9A, H15A M-CSF mutein was otherwise not significantly different from the parental M-CSF α , including crystallizability and space group (see Example 12) we believe the decrease in biological activity is not due to gross deformation of structure but reflects an
- 15 alteration in important M-CSF receptor contacts.

Using essentially the same methodology, two M-CSF muteins contacting singly substituted histidines at residues 9 and 15 were generated (e.g. H9A and H15A). The H9A construct utilized LF80:

- 20 5'-CAGGAGAAAGCTTATGTCTGAATATTGTAGCGCCATGATTGGGAGTGGACACCTGCAG-3' (SEQ ID NO 3); and LF73 (described in Example 9) as the PCR primers. The H15A construct utilized LF81:

- 25 5'-CAGGAGAAAGCTTATGTCTGAATATTGTAGCCACATGATTGGGAGTGGAGCCCTGCAG-3' (SEQ ID NO. 4); and LF 73 (described in Example 9) as PCR primers. Biological assay of the purified muteins immediately following the refolding step described
- above showed approximate biological specific activities as follows: 4×10^6 U/mg for H9A and less than 3×10^3 U/mg for H15A, in an assay which the parental M-CSF construct displayed 8×10^7 U/mg. This information, combined with that described above, suggests that H15A as well as possible H9A represents contacts that are important for M-CSF receptor binding. Nearby solvent accessible residues such
- 30 as Y6 and S13 (*see also* Table 1) may also represent M-CSF receptor contact residues. Non-proteinaceous mimics of the side chains of H9, H15, and nearby

solvent accessible side chains may represent M-CSF agonists or antagonists. Such residues should be left unchanged in M-CSF mutein constructs designed to retain full M-CSF receptor binding but to have M-CSF antagonist properties because they lack significant M-CSF bioactivity. Homodimers of muteins that retain full receptor-binding ability and display significantly reduced bioactivity should represent M-CSF antagonists. M-CSF muteins that are greatly decreased in both M-CSF bioactivity and receptor binding ability (such as H15A) may generally be useful in M-CSF immunoassay applications and might represent useful therapeutic agents for patients having auto-antibodies to M-CSF.

10

Example 11

Preparation of Q20A, V78K M-CSF Muteins

Using essentially the same methodology described in Example 10, a double mutant of M-CSF (Q20A, V78K) was constructed to test the importance of solvent accessible residues in the central portion of helices A and C. The following PCR primers were used.

15 LF63:

5'-AGGAGAAAGCTTATGTCTGAATATTGTAGCCACATGATTGGGAGTGGACACCTGCAGTCTCTGGCTCGGCTG-3' (SEQ ID NO. 5)

LF64:

20 5'-GGACGCAGGCCTTGTCATGCTCTTCATAATCCTTGGTGAAGCAGCTCTTCAGCCTCAAAGAGAGTTCCTGCAGCTGTTTAATGGC-3' (SEQ ID NO. 6)

The resulting mutein was expressed, refolded, purified, and assayed as described in Example 8. The specific biological activity was 1.4×10^7 U/mg, approximately 8-10 fold lower than that of the parental M-CSF α reference standard.

25 The receptor binding activity of this mutein was also decreased.

This result again supports the prediction of over crystallographic study of truncated M-CSF α which concluded that important M-CSF receptor contact

residues exist among the solvent accessible residues in helices A and/or C and/or D. Certain of these mutations will, as we have shown, have lower biological activity and lower M-CSF receptor-binding ability. Some may have lower biological activity without a decrease in receptor-binding ability. Some may have increased biological activity and receptor binding ability, and some may have no affect on either.

Two examples of the latter are Q17A, R21A (produced using PCR primers LF72:

5'-TTGTAGCCACATGATTGGGAGTGGACACCTGGCGTCTCTGCAGGCGCTGATTGAC-3' and LF73 (described in Example 9) and E115A, N119A (produced using LF75:
5'-CATGACAAGGCCTGCGTCCGAACCTTTCTATGAGACACCTCTCCAGTTGCTGGCGAAGG
TCAAGGCTGTCTTTAATG-3' (SEQ ID NO. 7); and
LF79: 5'-GGATCAGGATCCCTCGGACTGCCTCTC-3' (SEQ ID NO. 8)).

Both of these constructs changed side chain properties of solvent-accessible amino acids in the areas of interest but did not affect biological specific activity, compared to the parental reference molecule. These results indicate that residues Q17, R21, E115, and N119 do not need to be altered in muteins designed to have M-CSF agonist or antagonist activity. In fact, to minimize the likelihood of antibody formation to potentially administered M-CSF-based proteinaceous drugs, it is desirable to retain the solvent-accessible parental M-CSF residues (to resemble the native molecule) whenever possible.

The retained activity of the muteins including changes at Q17, R21, E115, and N119 does not rule out large affects on activity contributed by nearby residues (such as H15). In fact, the regions we have altered are predicted by the crystal structure to be important for receptor binding and/or signaling. Antagonistic M-CSF muteins may require use of multiple residue changes per mutein or use of heterodimeric molecules containing one or more mutations in each polypeptide chain, since M-CSF residues important in receptor signaling are believed to be composed of discontinuous regions of M-CSF.

Example 12

Formation of M-CSF Heterodimers Having Decreased Receptor-Binding Ability and/or Decreased Biological Specific Activity

M-CSF can be folded *in vitro* to generate fully active heterodimers, as shown in Example 8. By making heterodimers of M-CSF which incorporate M-CSF muteins with altered M-CSF signaling ability, it should be possible to generate antagonists of M-CSF useful for treatment of patients with M-CSF mediated diseases. To generate a heterodimer containing one subunit of M-CSF α N'3/C'158, H9A/H15A and one subunit of M-CSF β N'3/C'221, C157S/C159S, each mutein was expressed in *E. coli* and purified separately by DEAE Sepharose under denaturing and reducing conditions as described in Example 8. The two muteins subunits were mixed together prior to refolding to generate a solution containing a final mutein molar ratio of 1:1, then this solution was diluted to 0.2 mg/ml with refolding buffer as described in Example 8. Following refolding, the heterodimeric molecule was separated from the homodimers by two consecutive passes over a Phenyl TSK-5-PW HPLC column as described in Example 8. No contaminants were detected when the purified heterodimer preparation was examined by non-reduced SDS-PAGE or size exclusion HPLC using a BioSil SEC250 column (BioRad).

The purified heterodimer was submitted to the NFS60 cell based bioassay described in Example 8. The calculated specific activity was 2.9×10^6 U/mg which correlated to a 35-fold reduction as compared to the activity of the parental M-CSF heterodimer described in Example 10. The relative binding affinity to cell surface M-CSF receptor was measured by radioligand displacement in which the displacement of ^{125}I -M-CSF from an M-CSF receptor by an M-CSF mutein was measured using methods well known in the art. In brief, the following were added in a final volume of 100 μl in each well of a 96-well cell culture plate: approximately 80,000 cpm of purified recombinant human M-CSF labeled with ^{125}I (using Iodobeads as described by the manufacturer, Pierce, Rockford, IL), 300,000 NFS-60 cells that had been washed and then grown for 18 hours in growth medium minus the normal

maintenance level of M-CSF, plus unlabeled M-CSF that had been serially diluted in the same medium. The plates were incubated at 4°C for 20 hours and the cells were collected on glass-fiber filters. Maximum binding was measured in the absence of unlabeled M-CSF and non-specific binding was measured in the presence of 1000-fold greater concentration of unlabeled M-CSF (compared to labeled M-CSF). The concentration of M-CSF required to inhibit 50% of the labeled M-CSF binding to the cells (IC_{50}) was used to determine differences in affinity. Results are expressed as percent displacement of radioactive M-CSF versus mutein concentration (Figure 7). The IC_{50} of the heterodimer (Fig. 7 closed squares) was reduced 30-fold to about 500 pM as compared to an IC_{50} of about 17 pM for M-CSF α N'3/C"158 (158) (Fig. 7 closed squares). The similarity between the reduction in specific activity and receptor affinity of the heterodimer indicates that the reduction in bioactivity was due to decreased receptor-binding ability. Similarly, the binding affinities of the Q20A/V78KF (Fig. 7 open circles) and H9A/H15A (open squares) muteins were also measured in this radioligand displacement assay. The Q20A/V78K mutein had an IC_{50} of about 100 pM and the H9A/H15A mutein has an IC_{50} of about 1 μ M, correlating to decreased binding affinities of 5-fold and 50,000-fold, respectively. For each mutein, the reduction in receptor affinity was similar to the reduction in specific activity, again indicating that the reduction in bioactivity was due to reduced receptor-binding ability.

Example 13

Crystallization and Characterization Of M-CSF H9A, H15A Muteins

The H98, H15A mutein described in Example 9 was crystallized using the hanging drop method described in Examples 1 and 2 using the following buffer conditions: 30% polyethylene glycol 4000; 100 mM $Li_2 SO_4$; and 100 mM Tris pH 8.5. The crystals produced under these conditions were rhombohedral prisms having dimensions of 0.7 mm x 0.2 mm x 0.2 mm. X-ray crystallographic analysis using precession photographs showed crystals in the P2, 2, 2 space group with cell

dimensions of $a = 33.99$, $b = 65.37$, $c = 159.90$, $d = 90$, $e = 90$, and $f = 90$ angstroms and diffract to a nominal resolution of 3 angstroms. These physical properties are essentially the same as those observed for the parental $N\Delta 3C\Delta 158$ M-CSF α molecule and suggests that the biological effects of the H9A, H15A alterations
5 are not the consequence of gross global alterations in M-CSF structure, but rather are the result of altered side chains that are important in interacting with the M-CSF receptor. Alteration of those histidine side chains may have affected receptor binding by changing atoms that interact with, stabilize or facilitate receptor binding or changes in receptor conformation. Changes such as H15A may also have affected
10 these functions by altering the position of the nearby side chain in M-CSF, most likely in the A and/or C helix regions.

The foregoing examples are presented by way of example and are not intended to limit the scope of the invention as set forth in the appended claims.

APPENDIX 1

ATOM	10	CA	SER	4	63.753	80.590	222.385	1.00	58.89
ATOM	13	CA	GLU	5	64.883	77.664	219.972	1.00	59.56
ATOM	23	CA	TYR	6	62.285	76.840	217.324	1.00	54.54
ATOM	37	CA	CYS	7	63.509	80.109	215.834	1.00	54.96
ATOM	44	CA	SER	8	66.853	79.529	214.160	1.00	54.41
ATOM	52	CA	HIS	9	65.466	77.798	211.053	1.00	55.11
ATOM	65	CA	MET	10	61.857	78.767	211.073	1.00	50.66
ATOM	74	CA	ILE	11	62.173	80.905	207.970	1.00	48.31
ATOM	83	CA	GLY	12	63.487	78.618	205.354	1.00	52.83
ATOM	88	CA	SER	13	64.609	79.967	201.952	1.00	53.13
ATOM	96	CA	GLY	14	61.665	78.009	200.514	1.00	46.05
ATOM	101	CA	HIS	15	59.455	80.924	201.933	1.00	40.25
ATOM	114	CA	LEU	16	62.210	83.200	200.992	1.00	41.58
ATOM	123	CA	GLN	17	62.153	82.266	197.328	1.00	46.17
ATOM	135	CA	SER	18	58.378	81.868	197.227	1.00	46.55
ATOM	143	CA	LEU	19	58.280	85.544	198.199	1.00	44.75
ATOM	152	CA	GLN	20	60.814	86.044	195.458	1.00	39.32
ATOM	164	CA	ARG	21	58.457	84.718	192.736	1.00	36.91
ATOM	181	CA	LEU	22	55.610	86.694	194.252	1.00	38.27
ATOM	190	CA	ILE	23	57.676	89.822	193.465	1.00	34.84
ATOM	199	CA	ASP	24	58.677	88.274	190.086	1.00	31.50
ATOM	208	CA	SER	25	55.086	87.802	188.978	1.00	31.73
ATOM	216	CA	GLN	26	54.284	91.540	189.154	1.00	33.24
ATOM	228	CA	MET	27	53.961	93.634	186.082	1.00	34.82
ATOM	237	CA	GLU	28	56.227	96.566	186.089	1.00	36.54
ATOM	247	CA	THR	29	53.758	99.303	186.920	1.00	44.80
ATOM	256	CA	SER	30	54.590	102.747	188.323	1.00	49.40
ATOM	264	CA	CYS	31	51.427	103.423	190.046	1.00	43.03
ATOM	271	CA	GLN	32	52.166	104.336	193.613	1.00	33.74
ATOM	283	CA	ILE	33	50.430	102.961	196.634	1.00	32.48
ATOM	292	CA	THR	34	50.240	104.213	200.133	1.00	38.94
ATOM	301	CA	PHE	35	51.291	102.173	203.199	1.00	39.16
ATOM	313	CA	GLU	36	52.707	102.849	206.761	1.00	32.09
ATOM	323	CA	PHE	37	56.073	101.358	207.299	1.00	25.78

ATOM	335	CA	VAL	38	59.082	101.751	209.490	1.00	34.46
ATOM	343	CA	ASP	39	61.044	104.798	208.714	1.00	44.03
ATOM	352	CA	GLN	40	64.648	103.690	208.314	1.00	54.20
ATOM	364	CA	GLU	41	65.924	107.142	209.332	1.00	52.01
ATOM	374	CA	GLN	42	63.934	107.629	212.631	1.00	44.26
ATOM	386	CA	LEU	43	64.770	104.161	213.955	1.00	45.54
ATOM	395	CA	ALA	44	68.126	102.952	212.789	1.00	51.53
ATOM	401	CA	ASP	45	69.175	100.197	215.232	1.00	47.92
ATOM	410	CA	PRO	46	68.861	97.054	213.098	1.00	45.27
ATOM	417	CA	VAL	47	67.352	94.587	215.613	1.00	39.92
ATOM	425	CA	CYS	48	64.862	97.220	216.692	1.00	36.44
ATOM	432	CA	TYR	49	64.089	98.158	213.105	1.00	37.39
ATOM	446	CA	LEU	50	63.436	94.474	212.189	1.00	36.15
ATOM	455	CA	LYS	51	61.603	94.029	215.472	1.00	38.66
ATOM	468	CA	LYS	52	59.322	96.862	214.367	1.00	41.72
ATOM	481	CA	ALA	53	59.229	96.177	210.534	1.00	35.81
ATOM	487	CA	PHE	54	58.096	92.701	211.432	1.00	40.82
ATOM	499	CA	LEU	55	55.236	93.733	213.614	1.00	42.59
ATOM	508	CA	LEU	56	54.205	95.869	210.697	1.00	44.24
ATOM	517	CA	VAL	57	54.596	93.257	207.992	1.00	35.10
ATOM	525	CA	GLN	58	51.646	91.431	209.628	1.00	39.91
ATOM	537	CA	ASP	59	49.277	94.254	208.663	1.00	39.60
ATOM	546	CA	ILE	60	50.755	94.729	205.185	1.00	37.05
ATOM	555	CA	MET	61	50.303	90.932	204.676	1.00	33.86
ATOM	564	CA	GLU	62	46.766	90.931	206.028	1.00	48.10
ATOM	574	CA	ASP	63	45.602	94.198	204.286	1.00	49.51
ATOM	583	CA	THR	64	47.659	95.244	201.187	1.00	42.65
ATOM	592	CA	MET	65	49.106	92.155	199.603	1.00	37.90
ATOM	601	CA	ARG	66	45.646	91.249	198.376	1.00	39.50
ATOM	618	CA	PHE	67	45.516	88.245	195.824	1.00	35.13
ATOM	630	CA	ARG	68	42.655	86.013	194.326	1.00	44.68
ATOM	647	CA	ASP	69	41.727	83.454	196.865	1.00	47.42
ATOM	656	CA	ASN	70	43.117	80.057	195.984	1.00	44.47
ATOM	667	CA	THR	71	45.873	81.088	193.518	1.00	34.80

ATOM	676	CA	PRO	72	49.607	80.251	194.114	1.00	29.78
ATOM	683	CA	ASN	73	50.307	83.745	195.418	1.00	28.47
ATOM	694	CA	ALA	74	47.469	83.922	197.953	1.00	23.24
ATOM	700	CA	ILE	75	48.690	80.431	199.144	1.00	22.87
ATOM	709	CA	ALA	76	52.212	81.892	199.669	1.00	29.47
ATOM	715	CA	ILE	77	50.816	84.796	201.668	1.00	37.21
ATOM	724	CA	VAL	78	48.902	82.158	203.857	1.00	33.69
ATOM	732	CA	GLN	79	52.207	80.382	204.466	1.00	33.36
ATOM	744	CA	LEU	80	54.040	83.518	205.449	1.00	32.55
ATOM	753	CA	GLN	81	51.152	84.517	207.743	1.00	35.07
ATOM	765	CA	GLU	82	51.541	81.115	209.443	1.00	37.26
ATOM	775	CA	LEU	83	55.367	81.348	209.619	1.00	35.10
ATOM	784	CA	SER	84	54.960	84.840	211.066	1.00	39.64
ATOM	792	CA	LEU	85	52.608	83.541	213.704	1.00	42.55
ATOM	801	CA	ARG	86	54.984	80.903	215.121	1.00	42.96
ATOM	818	CA	LEU	87	57.916	83.317	214.670	1.00	45.11
ATOM	827	CA	LYS	88	56.504	85.876	217.138	1.00	50.71
ATOM	840	CA	SER	89	57.621	83.610	219.825	1.00	54.58
ATOM	848	CA	CYS	90	61.211	84.524	219.014	1.00	46.79
ATOM	855	CA	PHE	91	60.235	88.070	219.743	1.00	46.37
ATOM	867	CA	THR	92	59.619	89.812	223.026	1.00	52.01
ATOM	876	CA	ALA	93	56.916	92.445	223.523	1.00	56.09
ATOM	882	CA	ASP	94	57.914	96.123	223.998	1.00	59.35
ATOM	891	CA	TYR	95	55.685	99.100	225.194	1.00	66.22
ATOM	905	CA	GLU	96	52.401	100.066	223.612	1.00	65.51
ATOM	915	CA	GLU	97	53.343	103.890	223.182	1.00	62.60
ATOM	925	CA	HIS	98	56.046	102.712	220.836	1.00	58.47
ATOM	938	CA	ASP	99	53.422	100.733	218.887	1.00	59.97
ATOM	947	CA	LYS	100	52.950	103.470	216.162	1.00	57.00
ATOM	960	CA	ALA	101	56.259	105.270	216.975	1.00	48.82
ATOM	966	CA	CYS	102	58.861	105.450	214.154	1.00	42.41
ATOM	973	CA	VAL	103	56.175	104.773	211.621	1.00	33.87
ATOM	981	CA	ARG	104	55.993	106.851	208.456	1.00	42.76
ATOM	998	CA	THR	105	53.577	106.853	205.501	1.00	38.00

ATOM	1007	CA	PHE	106	54.931	105.944	202.136	1.00	33.48
ATOM	1019	CA	TYR	107	53.853	106.546	198.455	1.00	34.28
ATOM	1033	CA	GLU	108	55.765	103.906	196.543	1.00	33.88
ATOM	1043	CA	THR	109	55.266	101.309	193.854	1.00	36.94
ATOM	1052	CA	PRO	110	53.745	97.857	194.657	1.00	29.84
ATOM	1059	CA	LEU	111	57.132	96.374	193.761	1.00	35.66
ATOM	1068	CA	GLN	112	58.991	98.756	196.160	1.00	33.48
ATOM	1080	CA	LEU	113	56.612	97.691	198.912	1.00	24.26
ATOM	1089	CA	LEU	114	57.253	93.932	198.091	1.00	30.75
ATOM	1098	CA	GLU	115	61.063	94.385	197.984	1.00	39.68
ATOM	1108	CA	LYS	116	60.805	95.824	201.526	1.00	39.24
ATOM	1121	CA	VAL	117	58.669	92.840	202.743	1.00	32.46
ATOM	1129	CA	LYS	118	61.312	90.620	201.111	1.00	37.34
ATOM	1142	CA	ASN	119	64.231	92.316	202.959	1.00	39.46
ATOM	1153	CA	VAL	120	62.470	92.033	206.370	1.00	32.07
ATOM	1161	CA	PHE	121	61.935	88.268	206.034	1.00	26.35
ATOM	1173	CA	ASN	122	65.404	88.049	204.416	1.00	36.04
ATOM	1184	CA	GLU	123	67.372	89.816	207.138	1.00	40.82
ATOM	1194	CA	THR	124	65.331	88.435	210.122	1.00	41.72
ATOM	1203	CA	LYS	125	66.456	85.000	208.648	1.00	43.07
ATOM	1216	CA	ASN	126	70.010	86.339	208.179	1.00	48.89
ATOM	1227	CA	LEU	127	70.000	87.300	211.903	1.00	47.97
ATOM	1236	CA	LEU	128	68.315	84.194	213.435	1.00	47.21
ATOM	1245	CA	ASP	129	71.086	82.134	211.864	1.00	50.11
ATOM	1254	CA	LYS	130	73.448	84.146	214.117	1.00	52.04
ATOM	1267	CA	ASP	131	71.442	84.303	217.451	1.00	49.58
ATOM	1276	CA	TRP	132	67.946	82.685	218.322	1.00	53.33
ATOM	1292	CA	ASN	133	67.568	85.178	221.086	1.00	59.61
ATOM	1303	CA	ILE	134	68.572	88.447	219.305	1.00	49.82
ATOM	1312	CA	PHE	135	64.945	89.516	219.045	1.00	54.55
ATOM	1324	CA	SER	136	64.745	89.689	222.796	1.00	62.26
ATOM	1332	CA	LYS	137	66.563	93.119	222.569	1.00	54.38
ATOM	1345	CA	ASN	138	64.785	95.734	224.504	1.00	58.71
ATOM	1356	CA	CYS	139	63.902	98.337	222.050	1.00	51.21

ATOM	1363	CA	ASN	140	61.704	100.578	224.157	1.00	50.25
ATOM	1374	CA	ASN	141	64.219	103.338	224.061	1.00	58.30
ATOM	1385	CA	SER	142	65.158	102.992	220.352	1.00	52.18
ATOM	1393	CA	PHE	143	61.498	103.434	219.645	1.00	45.87
ATOM	1405	CA	ALA	144	61.829	106.488	221.831	1.00	47.49
ATOM	1411	CA	GLU	145	64.873	108.022	220.050	1.00	49.94
ATOM	1421	CA	CYS	146	62.489	108.299	217.017	1.00	53.65
ATOM	1428	CA	SER	147	60.005	111.197	217.009	1.00	57.45
ATOM	1436	CA	SER	148	57.163	110.805	214.395	1.00	59.53
ATOM	1444	CA	ALA	149	54.284	108.658	214.945	1.00	59.20
ATOM	1450	CA	GLY	150	51.655	108.783	212.248	1.00	61.95
ATOM	1455	CA	HIS	151	48.764	106.945	210.680	1.00	69.20
ATOM	1468	CA	GLU	152	46.475	107.377	207.803	1.00	76.61
ATOM	1478	CA	ALA	153	45.813	106.188	204.264	1.00	78.85
ATOM	1492	CA	SER	404	43.875	81.916	155.536	1.00	38.68
ATOM	1495	CA	GLU	405	41.939	78.947	156.798	1.00	43.57
ATOM	1505	CA	TYR	406	44.549	78.204	159.422	1.00	41.97
ATOM	1519	CA	CYS	407	43.251	81.196	161.434	1.00	36.99
ATOM	1526	CA	SER	408	41.011	78.917	163.442	1.00	34.02
ATOM	1534	CA	HIS	409	43.988	77.122	165.021	1.00	40.02
ATOM	1547	CA	MET	410	46.155	79.994	165.895	1.00	36.48
ATOM	1556	CA	ILE	411	44.849	80.843	169.353	1.00	37.88
ATOM	1565	CA	GLY	412	44.508	77.692	171.141	1.00	39.09
ATOM	1570	CA	SER	413	43.275	77.480	174.756	1.00	45.21
ATOM	1578	CA	GLY	414	46.813	77.142	176.120	1.00	41.00
ATOM	1583	CA	HIS	415	47.238	80.801	175.208	1.00	38.01
ATOM	1596	CA	LEU	416	44.181	81.554	177.458	1.00	39.12
ATOM	1605	CA	GLN	417	45.501	79.575	180.501	1.00	39.79
ATOM	1617	CA	SER	418	48.547	81.846	180.317	1.00	36.95
ATOM	1625	CA	LEU	419	46.482	85.106	180.212	1.00	33.42
ATOM	1634	CA	GLN	420	44.564	83.708	183.162	1.00	38.37
ATOM	1646	CA	ARG	421	47.839	82.875	185.041	1.00	41.95
ATOM	1663	CA	LEU	422	49.151	86.514	184.405	1.00	33.04
ATOM	1672	CA	ILE	423	45.854	87.864	185.738	1.00	25.14

ATOM	1681	CA	ASP	424	45.824	85.549	188.861	1.00	32.93
ATOM	1690	CA	SER	425	49.394	86.494	189.904	1.00	30.92
ATOM	1698	CA	GLN	426	48.776	90.276	189.999	1.00	29.47
ATOM	1710	CA	MET	427	48.379	91.899	193.372	1.00	31.04
ATOM	1719	CA	GLU	428	44.978	93.418	193.655	1.00	42.94
ATOM	1729	CA	THR	429	45.965	96.999	194.423	1.00	48.52
ATOM	1738	CA	SER	430	43.656	99.720	193.224	1.00	48.67
ATOM	1746	CA	CYS	431	46.604	101.354	191.803	1.00	52.34
ATOM	1753	CA	GLN	432	45.813	102.789	188.396	1.00	50.77
ATOM	1765	CA	ILE	433	47.826	103.166	185.221	1.00	51.17
ATOM	1774	CA	THR	434	46.923	105.057	182.065	1.00	52.62
ATOM	1783	CA	PHE	435	46.641	103.293	178.672	1.00	50.53
ATOM	1795	CA	GLU	436	45.167	103.968	175.164	1.00	50.56
ATOM	1805	CA	PHE	437	42.643	101.271	174.193	1.00	46.56
ATOM	1817	CA	VAL	438	39.404	101.233	172.116	1.00	52.14
ATOM	1825	CA	ASP	439	36.342	103.107	173.476	1.00	59.69
ATOM	1834	CA	GLN	440	33.750	100.386	173.243	1.00	63.82
ATOM	1846	CA	GLU	441	30.764	102.682	173.229	1.00	64.79
ATOM	1856	CA	GLN	442	31.810	104.569	170.091	1.00	59.01
ATOM	1868	CA	LEU	443	32.994	101.521	168.139	1.00	55.78
ATOM	1877	CA	LYS	444	30.279	99.137	169.086	1.00	54.82
ATOM	1890	CA	ASP	445	30.862	96.668	166.093	1.00	51.31
ATOM	1899	CA	PRO	446	32.144	93.267	167.135	1.00	47.34
ATOM	1906	CA	VAL	447	34.363	92.592	164.111	1.00	41.42
ATOM	1914	CA	CYS	448	35.680	96.184	163.845	1.00	39.44
ATOM	1921	CA	TYR	449	36.273	96.949	167.607	1.00	40.33
ATOM	1935	CA	LEU	450	38.224	93.580	167.624	1.00	30.87
ATOM	1944	CA	LYS	451	39.989	94.664	164.462	1.00	34.49
ATOM	1957	CA	LYS	452	40.962	97.918	166.163	1.00	35.38
ATOM	1970	CA	ALA	453	41.503	96.317	169.572	1.00	28.00
ATOM	1976	CA	PHE	454	44.012	93.845	168.164	1.00	32.56
ATOM	1988	CA	LEU	455	46.236	96.653	166.861	1.00	40.19
ATOM	1997	CA	LEU	456	46.402	98.605	170.114	1.00	42.45
ATOM	2006	CA	VAL	457	46.966	95.358	172.060	1.00	38.09

ATOM	2014	CA	GLN	458	50.298	95.232	170.161	1.00	48.02
ATOM	2026	CA	ASP	459	51.630	98.529	171.587	1.00	41.96
ATOM	2035	CA	ILE	460	50.230	97.686	175.001	1.00	25.82
ATOM	2044	CA	MET	461	52.409	94.542	175.085	1.00	33.69
ATOM	2053	CA	GLU	462	55.702	96.253	173.969	1.00	45.36
ATOM	2063	CA	ASP	463	55.069	99.093	176.249	1.00	48.41
ATOM	2072	CA	THR	464	52.771	98.555	179.186	1.00	41.63
ATOM	2081	CA	MET	465	52.743	94.854	180.171	1.00	42.05
ATOM	2090	CA	ARG	466	56.333	94.928	181.338	1.00	44.99
ATOM	2107	CA	PHE	467	57.618	91.923	183.501	1.00	38.72
ATOM	2119	CA	ARG	468	61.258	90.894	184.513	1.00	50.64
ATOM	2136	CA	ASP	469	63.046	88.868	181.895	1.00	53.46
ATOM	2145	CA	ASN	470	62.896	85.096	181.832	1.00	52.84
ATOM	2156	CA	THR	471	60.211	84.798	184.479	1.00	47.58
ATOM	2165	CA	PRO	472	57.055	82.652	183.744	1.00	46.82
ATOM	2172	CA	ASN	473	54.971	85.777	183.172	1.00	38.75
ATOM	2183	CA	ALA	474	57.431	87.402	180.828	1.00	37.27
ATOM	2189	CA	ILE	475	57.795	84.219	178.807	1.00	32.85
ATOM	2198	CA	ALA	476	54.021	84.034	178.455	1.00	27.98
ATOM	2204	CA	ILE	477	53.844	87.522	176.756	1.00	30.47
ATOM	2213	CA	VAL	478	56.690	86.495	174.201	1.00	30.40
ATOM	2221	CA	GLN	479	54.381	83.711	173.325	1.00	31.18
ATOM	2233	CA	LEU	480	51.419	86.038	172.893	1.00	27.44
ATOM	2242	CA	GLN	481	53.392	88.551	170.821	1.00	31.59
ATOM	2254	CA	GLU	482	54.572	85.683	168.506	1.00	29.90
ATOM	2264	CA	LEU	483	50.900	84.532	168.340	1.00	22.16
ATOM	2273	CA	SER	484	50.077	88.156	167.451	1.00	29.37
ATOM	2281	CA	LEU	485	52.410	87.880	164.490	1.00	32.70
ATOM	2290	CA	ARG	486	50.865	84.572	163.229	1.00	30.59
ATOM	2307	CA	LEU	487	47.396	86.383	163.343	1.00	31.02
ATOM	2316	CA	LYS	488	48.292	89.399	161.352	1.00	34.90
ATOM	2329	CA	SER	489	47.728	87.397	158.200	1.00	35.68
ATOM	2337	CA	CYS	490	44.112	87.134	159.417	1.00	35.03
ATOM	2344	CA	PHE	491	43.520	90.786	159.602	1.00	42.73

ATOM	2356	CA	THR	492	43.145	93.174	156.663	1.00	56.24
ATOM	2365	CA	ALA	493	44.877	96.467	156.617	1.00	57.56
ATOM	2371	CA	ASP	494	42.318	99.142	157.302	1.00	57.16
ATOM	2380	CA	ALA	495	42.063	102.909	156.516	1.00	62.14
ATOM	2386	CA	GLU	496	45.542	104.198	157.805	1.00	64.30
ATOM	2396	CA	GLU	497	44.589	107.547	159.382	1.00	70.67
ATOM	2406	CA	HIS	498	41.577	105.660	160.752	1.00	75.16
ATOM	2419	CA	ASP	499	44.400	103.329	161.968	1.00	72.11
ATOM	2428	CA	LYS	500	44.773	105.712	164.874	1.00	65.63
ATOM	2441	CA	ALA	501	41.040	106.628	165.617	1.00	55.63
ATOM	2447	CA	CYS	502	38.563	105.427	168.336	1.00	53.33
ATOM	2454	CA	VAL	503	41.155	105.639	171.100	1.00	52.46
ATOM	2462	CA	ARG	504	39.866	106.944	174.425	1.00	51.00
ATOM	2479	CA	THR	505	42.588	106.921	177.117	1.00	50.42
ATOM	2488	CA	PHE	506	41.955	104.935	180.331	1.00	43.64
ATOM	2500	CA	TYR	507	43.306	105.190	183.809	1.00	41.84
ATOM	2514	CA	GLU	508	42.651	101.611	185.218	1.00	39.35
ATOM	2524	CA	THR	509	44.156	99.096	187.597	1.00	41.67
ATOM	2533	CA	PRO	510	46.618	96.563	185.969	1.00	39.70
ATOM	2540	CA	LEU	511	44.134	93.600	186.416	1.00	42.72
ATOM	2549	CA	GLN	512	41.460	95.632	184.624	1.00	37.47
ATOM	2561	CA	LEU	513	43.895	96.148	181.765	1.00	27.02
ATOM	2570	CA	LEU	514	44.769	92.421	181.627	1.00	24.55
ATOM	2579	CA	GLU	515	41.076	91.327	181.688	1.00	31.93
ATOM	2589	CA	LYS	516	40.903	93.442	178.515	1.00	31.96
ATOM	2602	CA	VAL	517	43.981	91.949	176.692	1.00	26.46
ATOM	2610	CA	LYS	518	42.528	88.538	177.852	1.00	30.83
ATOM	2623	CA	ASN	519	39.106	89.397	176.432	1.00	37.62
ATOM	2634	CA	VAL	520	40.221	90.488	172.933	1.00	35.75
ATOM	2642	CA	PHE	521	42.191	87.109	172.662	1.00	25.45
ATOM	2654	CA	ASN	522	39.292	85.079	173.953	1.00	26.73
ATOM	2665	CA	GLU	523	36.870	86.722	171.437	1.00	33.04
ATOM	2675	CA	THR	524	39.260	86.923	168.369	1.00	29.70
ATOM	2684	CA	LYS	525	39.423	83.195	169.011	1.00	28.41

ATOM	2697	CA	ASN	526	35.624	82.663	169.124	1.00	34.46
ATOM	2708	CA	LEU	527	34.816	84.653	166.021	1.00	28.09
ATOM	2717	CA	LEU	528	37.617	82.902	164.068	1.00	25.62
ATOM	2726	CA	ASP	529	35.899	79.662	164.964	1.00	35.00
ATOM	2735	CA	LYS	530	32.659	81.068	163.537	1.00	37.54
ATOM	2748	CA	ASP	531	34.250	82.150	160.272	1.00	37.92
ATOM	2757	CA	TRP	532	38.053	82.453	159.573	1.00	40.02
ATOM	2773	CA	ASN	533	38.395	85.446	157.159	1.00	42.64
ATOM	2784	CA	ILE	534	35.840	87.437	159.393	1.00	39.27
ATOM	2793	CA	PHE	535	38.635	89.996	159.881	1.00	40.49
ATOM	2805	CA	SER	536	38.485	90.720	156.238	1.00	46.56
ATOM	2813	CA	LYS	537	35.844	93.371	157.102	1.00	45.61
ATOM	2826	CA	ASN	538	35.653	96.936	155.760	1.00	54.39
ATOM	2837	CA	CYS	539	35.786	98.626	159.004	1.00	50.55
ATOM	2844	CA	ASN	540	36.738	101.888	157.286	1.00	58.49
ATOM	2855	CA	ALA	541	33.143	102.934	157.648	1.00	61.90
ATOM	2861	CA	SER	542	32.854	101.855	161.326	1.00	56.62
ATOM	2869	CA	PHE	543	35.911	103.888	162.235	1.00	55.25
ATOM	2881	CA	ALA	544	33.885	107.102	161.741	1.00	61.94
ATOM	2887	CA	GLU	545	30.949	107.003	164.203	1.00	63.83
ATOM	2897	CA	CYS	546	33.917	107.418	166.572	1.00	61.90
ATOM	2904	CA	SER	547	33.751	110.878	168.150	1.00	66.22
ATOM	2912	CA	ALA	548	37.461	110.300	168.811	1.00	65.91

END

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANTS: CHIRON CORPORATION AND
THE REGENTS OF THE UNIVERSITY OF CALIFORNIA
- (ii) TITLE OF INVENTION: CRYSTALLIZATION OF M-CSF
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
 - (B) STREET: 6300 Sears Tower, 233 South Wacker Drive
 - (C) CITY: Chicago
 - (D) STATE: Illinois
 - (E) COUNTRY: USA
 - (F) ZIP: 60606-6402
- (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:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/896,512 CIP
 - (B) FILING DATE: 09-JUN-1992
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Clough, David W.
 - (B) REGISTRATION NUMBER: 36,107
 - (C) REFERENCE/DOCKET NUMBER: 31436
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 312/474-6300
 - (B) TELEFAX: 312/474-0448
 - (C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 56 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA

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

AGGTGTCTCA TAGAAAGTTC GGACGCAGGC CTTGTCATGC TCTTCATAAT CCTTGG

56

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 58 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

CAGGAGAAAG CTTATGTCTG AATATTGTAG CGCCATGATT GGGAGTGGAG CCCTGCAG 58

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 58 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

CAGGAGAAAG CTTATGTCTG AATATTGTAG CGCCATGATT GGGAGTGGAC ACCTGCAG 58

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 58 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

CAGGAGAAAG CTTATGTCTG AATATTGTAG CCACATGATT GGGAGTGGAG CCCTGCAG 58

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 72 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

AGGAGAAAGC TTATGTCTGA ATATTGTAGC CACATGATTG GGAGTGGACA CCTGCAGTCT 60

CTGGCTCGGC TG 72

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 85 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

GGACGCAGGC CTTGTCATGC TCTTCATAAT CCTTGGTGAA GCAGCTCTTC AGCCTCAAAG 60
AGAGTTCCTG CAGCTGTTTA ATGGC
85

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 76 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

CATGACAAGG CCTGCGTCCG AACTTTCTAT GAGACACCTC TCCAGTTGCT GGCGAAGGTC 60
AAGGCTGTCT TTAATG
76

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

GGATCAGGAT CCCTCGGACT GCCTCTC 27

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

GCGTACCATG GGCCCAGGAG TTCTGC

26

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

AGTCGAGGAT CCTCAATCCG GGGGATGCGT GTG

33

WHAT IS CLAIMED IS:

1. A composition comprising crystalline M-CSF, the M-CSF being a dimer of two M-CSF polypeptide monomers, wherein each monomer has substantially the same amino acid sequence as a polypeptide selected from the group consisting of
5 mature M-CSF α polypeptide, mature M-CSF β polypeptide, and mature M-CSF γ polypeptide.
2. The composition of claim 1 wherein the crystalline dimer has P2₁2₁2₁ symmetry.
3. The composition of claim 2 wherein the M-CSF is biologically
10 active when resolubilized.
4. The composition of claim 1 wherein the two M-CSF polypeptide monomers are disulfide-linked to each other.
5. The composition of claim 1 wherein each of the two M-CSF polypeptide monomers is an M-CSF α polypeptide.
- 15 6. The composition of claim 1 wherein each monomer has an amino acid sequence between about 145 and about 156 amino acids in length, and wherein said sequence has a carboxylic acid terminus located between about 145 and 156 residues measured from the amino terminus of a mature M-CSF polypeptide.
7. The composition of claim 6 wherein at least one of the
20 polypeptides contains residues 4 to 158 of mature M-CSF α polypeptide.
8. The composition of claim 1 wherein at least one of the M-CSF polypeptide monomers is unglycosylated.

9. A method of crystallizing a M-CSF α dimer having two polypeptide monomers, each containing 146 to 162 amino acids, the method comprising the steps of:

- 5 a) mixing a solution of the M-CSF α dimer with a precipitant, whereby an M-CSF α mixture is formed;
- b) precipitating crystalline M-CSF α dimer; and
- c) isolating the crystalline M-CSF α dimer.

10 10. The method of claim 9 wherein the precipitant comprises polyethylene glycol.

10 11. The method of claim 9 wherein the M-CSF dimer is produced from recombinant M-CSF isolated from a bacterial cell.

 12. The method of claim 9 wherein at least one of the polypeptide monomers is unglycosylated.

15 13. The method of claim 9 wherein at least one of the polypeptide monomers contains residues 4 to 158 of mature M-CSF α polypeptide.

 14. The method of claim 9 wherein the crystalline M-CSF α dimer is biologically active when resolubilized.

20 15. The method of claim 9 wherein the step of precipitating the M-CSF α crystal comprises equilibrating the M-CSF α dimer mixture with a precipitant solution.

 16. The method of claim 15 wherein the precipitant solution comprises a higher concentration of precipitant than the M-CSF α mixture.

17. The method of claim 15 wherein the step of equilibrating comprises applying the M-CSF α mixture to a surface and equilibrating the applied M-CSF α mixture with a reservoir of the precipitant solution.

18. The method of claim 9 wherein the step of precipitating the
5 crystalline M-CSF α dimer comprises a step of applying M-CSF α seed crystals to the M-CSF α dimer mixture.

19. The method of claim 9 wherein the step of precipitating the M-CSF α dimer comprises the step of altering the temperature of the M-CSF α mixture.

10 20. Crystalline M-CSF α (4-158)

21. The crystalline M-CSF of claim 20 comprising crystals of M-CSF having measurements of at least about 0.3 mm in at least two dimensions.

22. The crystalline M-CSF of claim 20 which is capable of
15 diffracting X-ray radiation to produce a diffraction pattern representing the three-dimensional structure of the M-CSF.

23. The crystalline M-CSF of claim 22 wherein the diffraction pattern represents the three-dimensional structure of a portion of the M-CSF α (4-158) to an accuracy of at least five angstroms, wherein the portion includes amino acid residues between residue 4 and about residue 153.

20 24. A method for determining a three-dimensional structure of an M-CSF dimer containing two M-CSF polypeptide monomers, each having between about 146 and 162 amino acids beginning from about residue 1 to about residue 5 of mature M-CSF, the method comprising:

- 5
- a) crystallizing the M-CSF dimer;
 - b) irradiating the crystalline M-CSF to obtain a diffraction pattern characteristic of the crystalline M-CSF; and
 - c) transforming the diffraction pattern into the three-dimensional structure of the M-CSF dimer.

25. A method of using a three-dimensional structure of M-CSF derived from an M-CSF crystal wherein the three-dimensional structure of M-CSF includes an M-CSF receptor-binding region, the method comprising identifying compounds having structures that interact with a receptor-binding region of the three-dimensional structure of M-CSF and function as an M-CSF agonist or antagonist.

10

26. The method of claim 25 wherein the three-dimensional structure of M-CSF includes alpha-carbon coordinates substantially the same as those of the structural information presented in Appendix 1.

- 15
27. A method of identifying M-CSF agonists or antagonists, the method comprising the following steps:
- a) crystallizing an M-CSF dimer to form at least one M-CSF crystal, the M-CSF dimer containing a group of amino acid residues defining an M-CSF receptor-binding region;
 - b) irradiating an M-CSF crystal produced by the procedure of step (a) to obtain a diffraction pattern of the M-CSF crystal;
 - c) determining the three-dimensional structure of M-CSF from the diffraction pattern, the three-dimensional structure including an M-CSF receptor-binding region; and
 - d) identifying an M-CSF agonist or antagonist compound having a three-dimensional structure that functionally duplicates essential M-CSF receptor-binding solvent accessible residues present in the
- 20
- 25

three-dimensional structure of the M-CSF receptor-binding region, said M-CSF agonist or antagonist having altered signal transduction capacity to M-CSF responsive cells.

28. The method of claim 27 wherein said solvent accessible residues
5 do not participate in formation of the M-CSF dimer interface.

29. A method for producing M-CSF antagonists comprising the steps of:

- 10 a) altering solvent accessible amino acid residues involved in signal transduction and wherein said alteration does not impair binding of M-CSF to the M-CSF receptor; and
b) testing said M-CSF antagonist for activity in an M-CSF bioassay.

30. The method of claim 29 wherein solvent accessible amino acids are altered by chemical modification.

15 31. The method of claim 29 wherein at least one and preferably fewer than five solvent accessible amino acids per M-CSF monomer are modified by amino acid substitution.

32. The method of claim 29 wherein said M-CSF is a heterodimer and wherein only one monomer has substituted solvent accessible amino acids and
20 wherein said solvent accessible amino acids are involved in signal transduction.

33. The method of claim 29 wherein said M-CSF is a heterodimer and wherein both monomers have different substituted solvent accessible amino acids, and wherein said solvent accessible amino acids are involved in signal transduction.

34. An isolated, purified, soluble and functional M-CSF receptor.

35. A method for screening M-CSF agonists and antagonists, said method comprising the steps of:

- a) contacting said agonist or antagonist with a soluble
5 functional M-CSF receptor; and
- b) measuring the interaction of said agonist or said antagonist with said receptor.

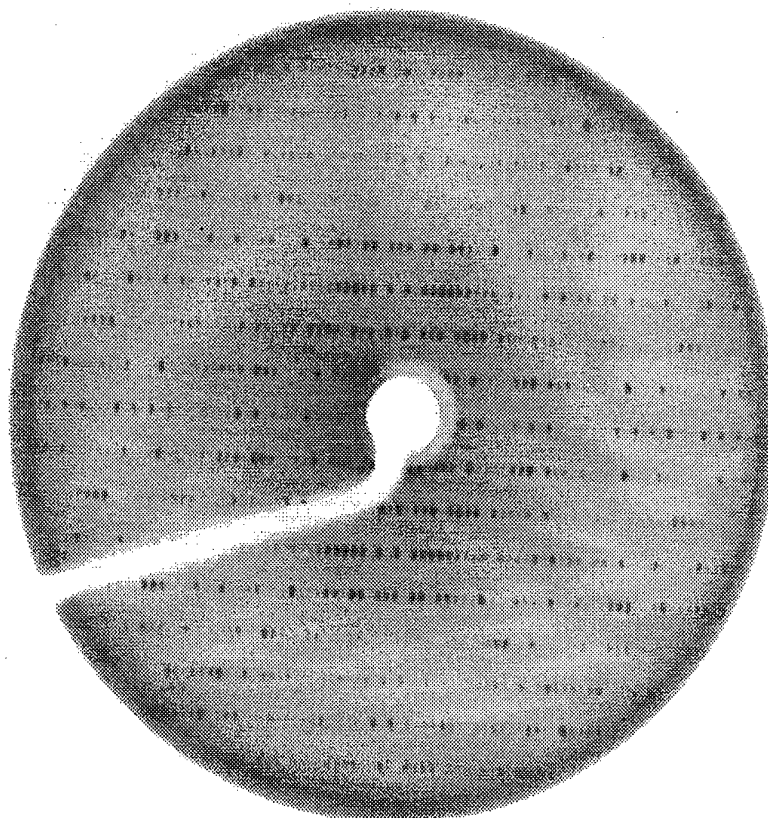


FIG. 1

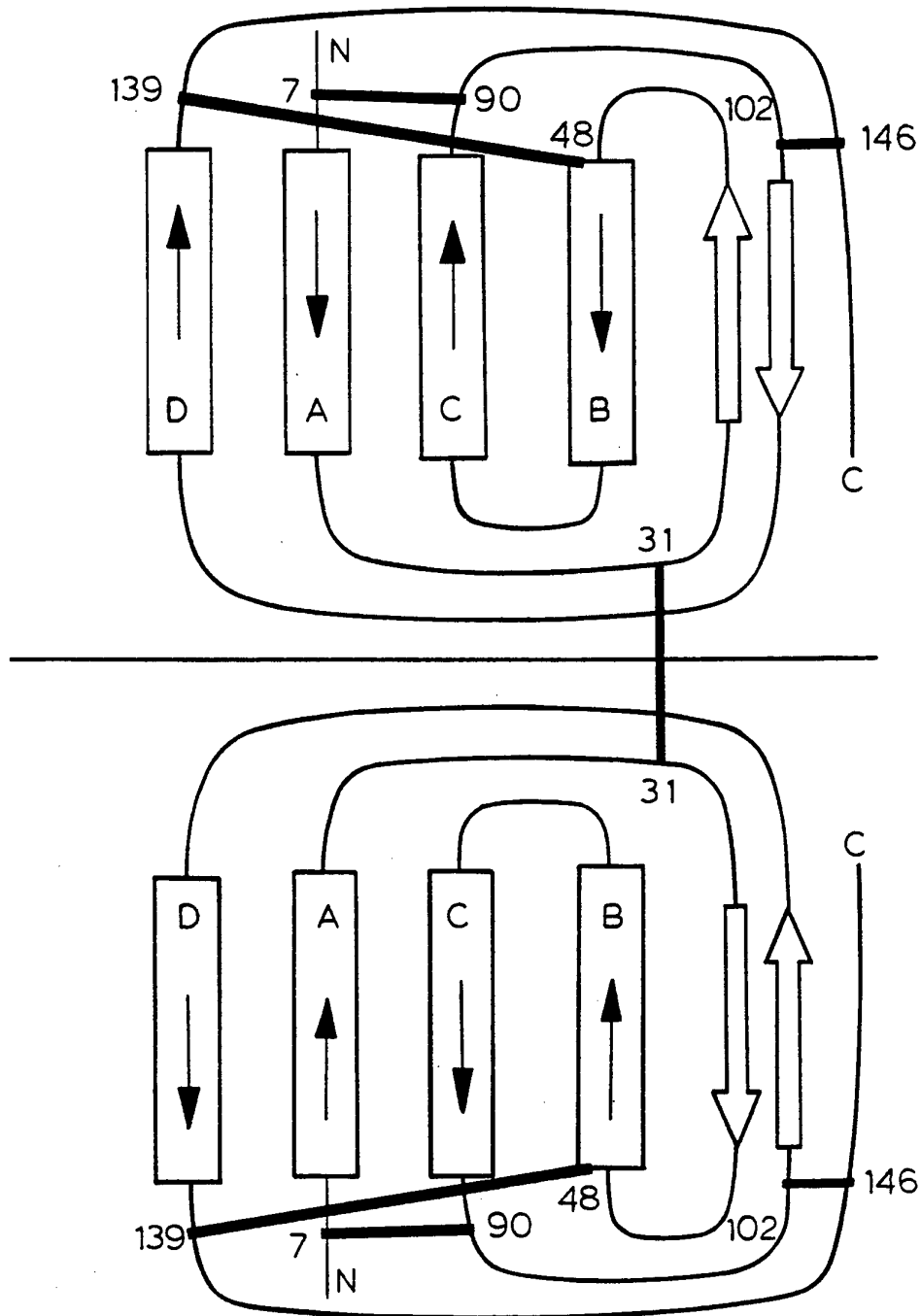


FIG. 2

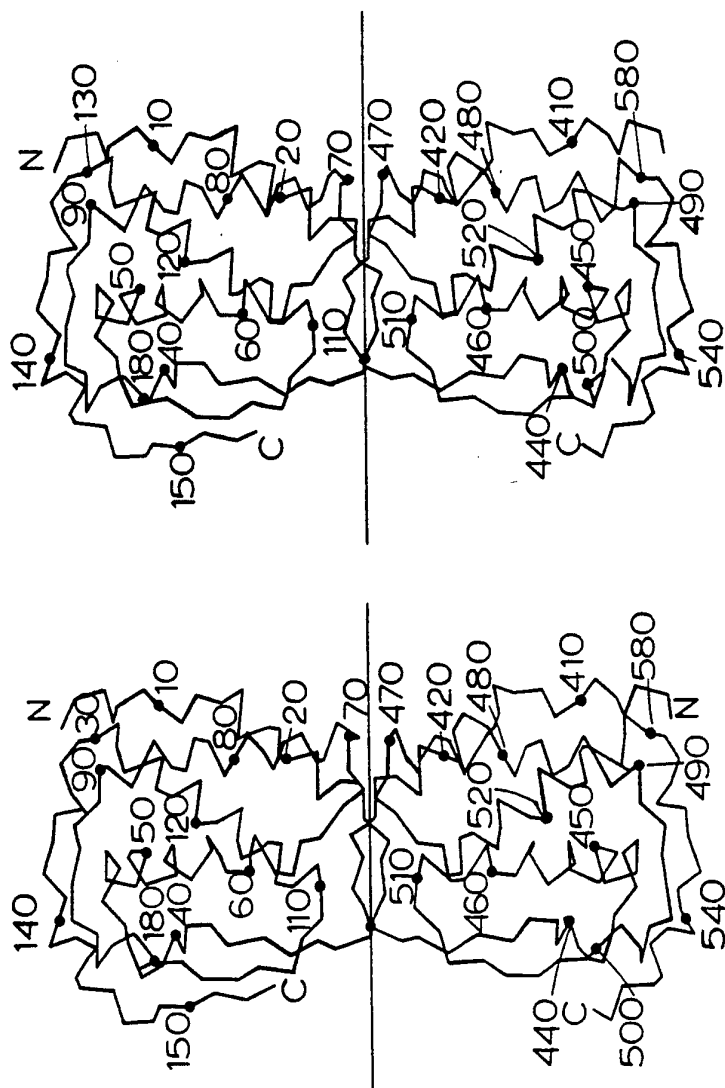


Fig. 3

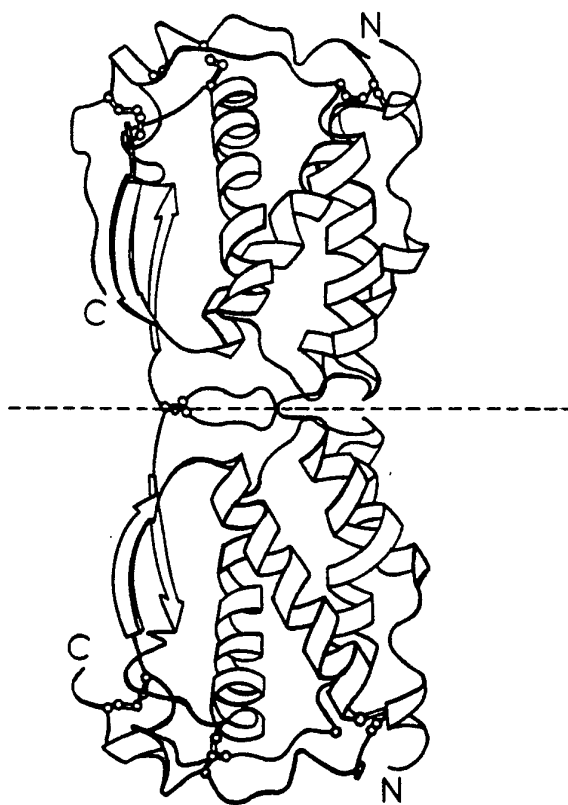


FIG. 4

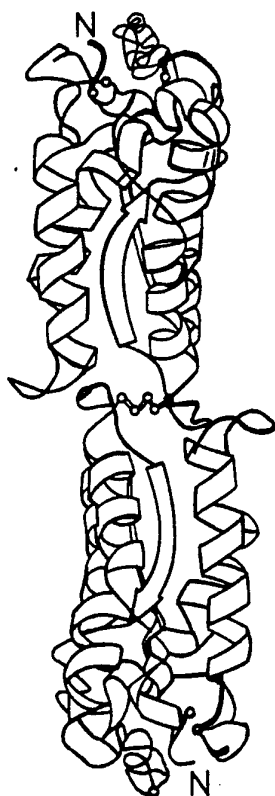


FIG. 4A

FIG. 5A

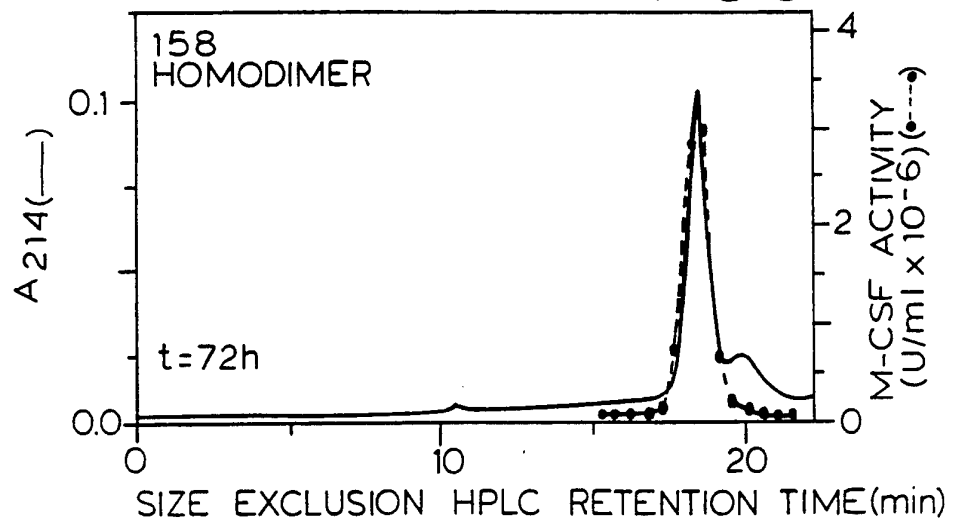


FIG. 5B

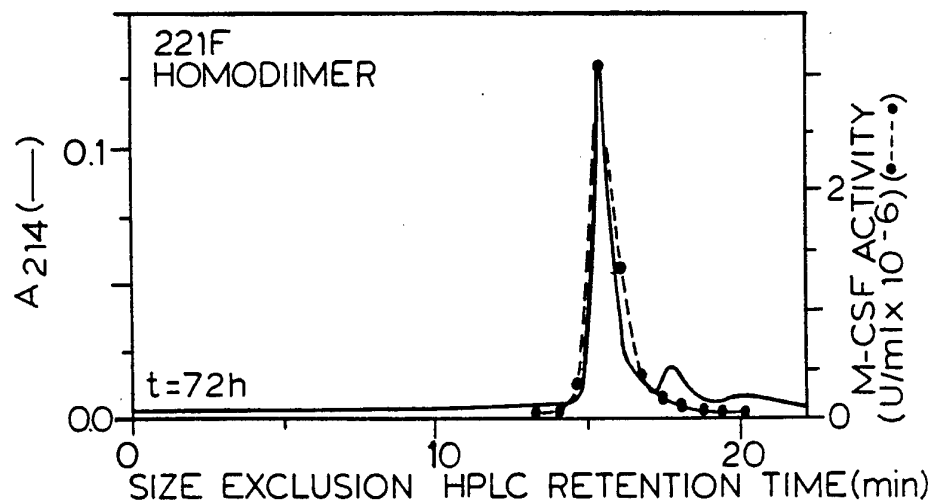
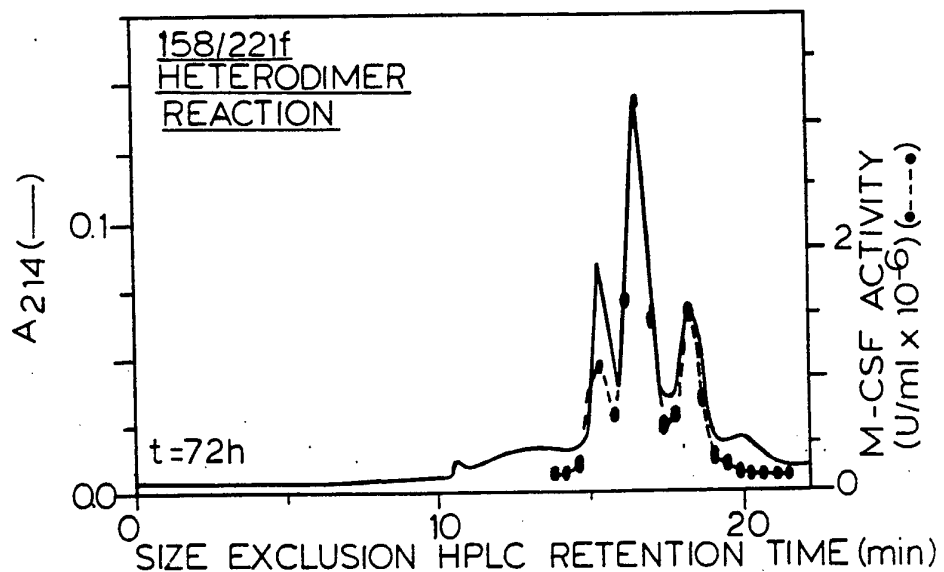


FIG. 5C



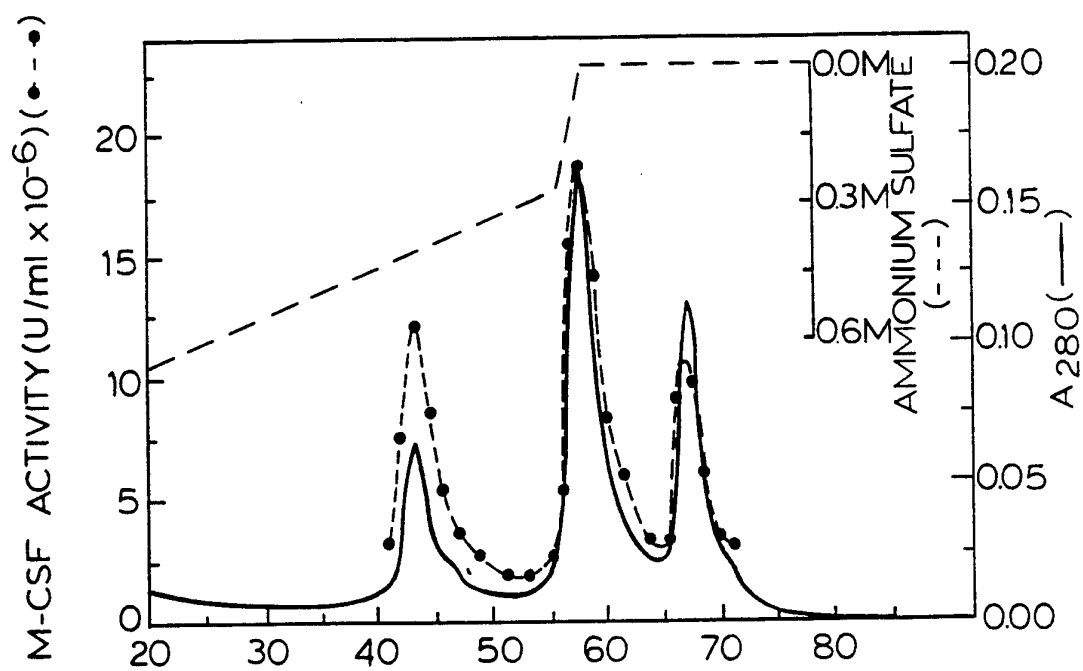


FIG. 6A

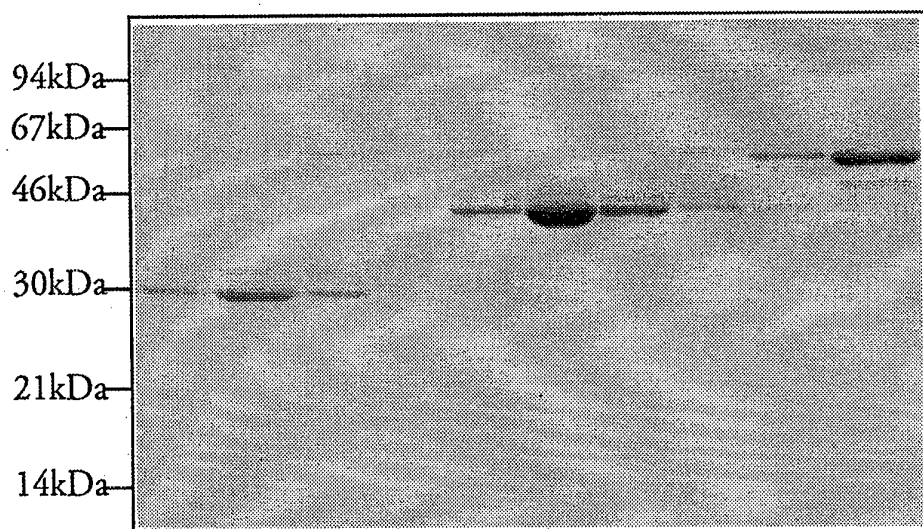


FIG. 6B

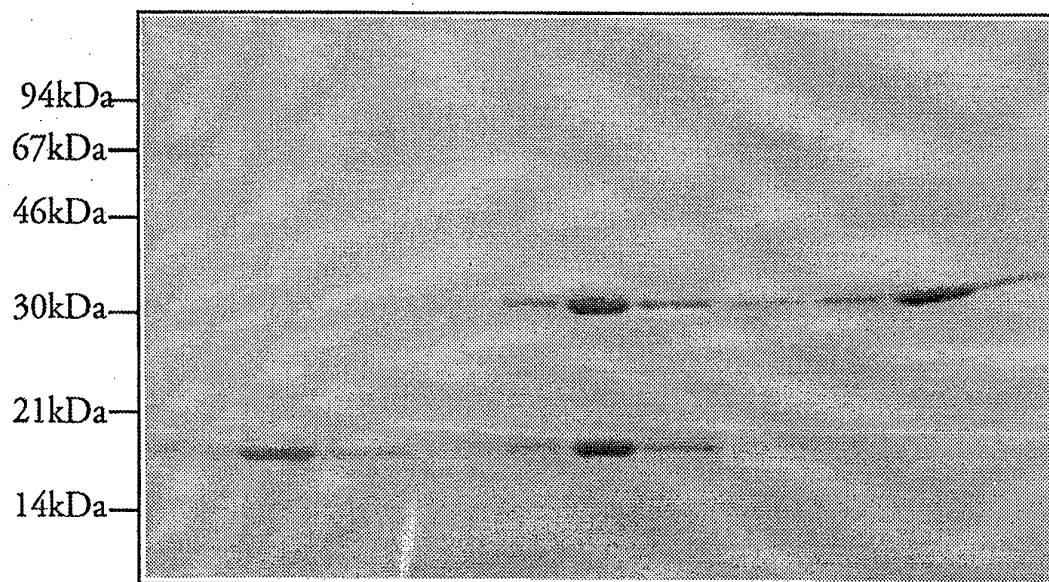


FIG. 6C

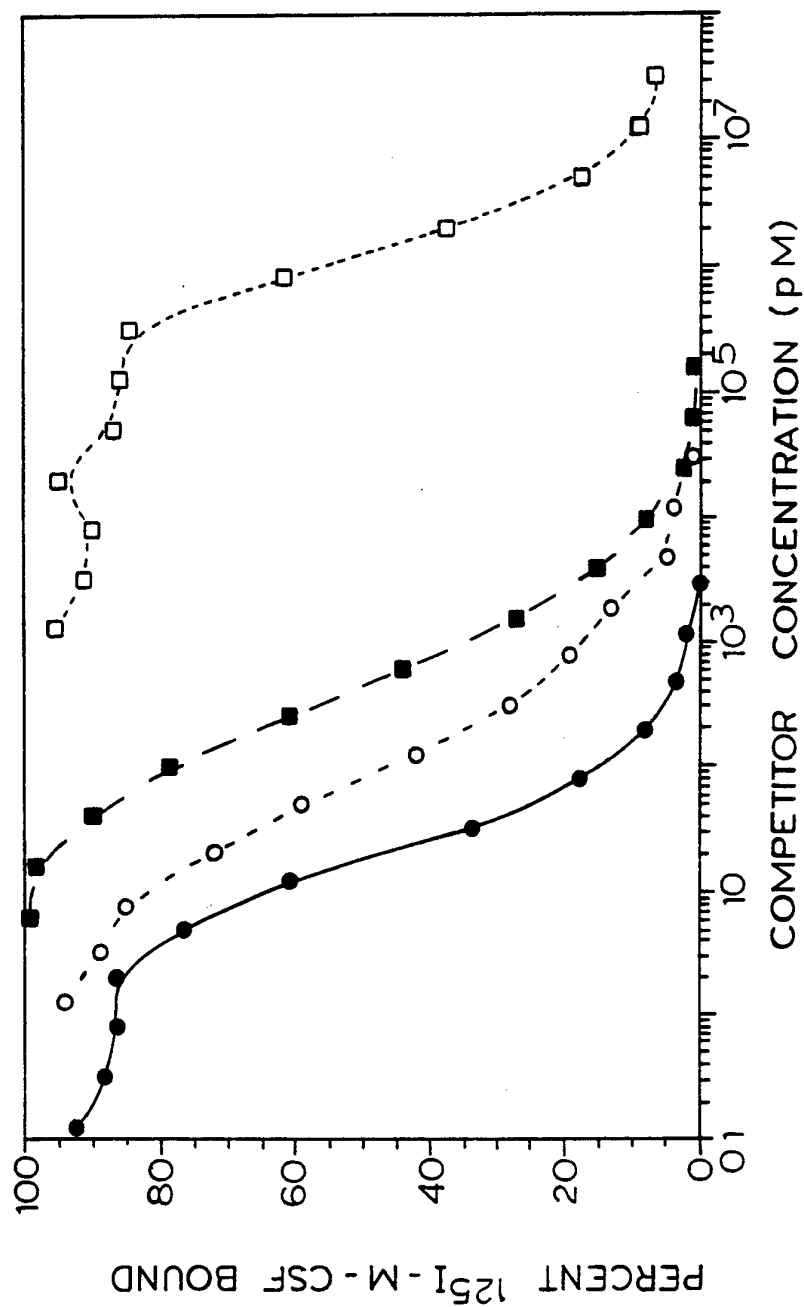


FIGURE 7

INTERNATIONAL SEARCH REPORT

PCT/US 93/05548

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5	C12N15/27; G01N33/68	C12P21/02; C07K13/00; C12N15/12
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	WO,A,8 808 003 (CETUS CORPORATION) 20 October 1988 * see the whole document especially examples 1-4, the claims * & US,A,4 929 700 (CETUS CORPORATION) 29 May 1990 cited in the application ---	1-24
Y	EP,A,0 402 070 (SCHERING CORPORATION) 12 December 1990 see the whole document ---	1-24
Y	R. K. SCOPES 'protein purification advanced texts in chemistry' 1987, SECOND EDITION. PAGES 297-321. SPRINGER-VERLAG, NEW YORK, US. see page 297 - page 301 ---	1-24
-/--		
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
12 OCTOBER 1993		22. 10. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		LE CORNEC N.D.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,A	WO,A,9 221 029 (GENENTECH , INC.) 26 November 1992 cited in the application see page 3 - page 5 see claims ---	25-33,35
Y	EP,A,0 344 796 (CHUGAI SEIYAKU KABUSHIKI KAISHA) 6 December 1989 * see the whole document especially page 3 lines 25-52 , example 2 * ---	1-24
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 251, no. 20, 25 October 1976, . BALTIMORE US pages 6300 - 6303 A. MCPHERSON, JR 'Crystallization of proteins from polyethylene glycol' cited in the application see the whole document ---	1-24
P,X	SCIENCE vol. 258, 20 November 1992, LANCASTER, PA US pages 1358 - 1362 J. PANDIT ET AL 'Three-dimensional structure of dimeric human recombinant Macrophage Colony-Stimulating Factor' see the whole document ---	1-24
A	SPORN M.B. ET ROBERTS A.B. 'peptide growth factors and their receptors, handbook of experimental pharmacology vol. 1; vol. 95/1. pages 667-698.' 1990 , SPINGER VERLAG , DE, BERLIN. see page 671 - page 689 ---	34
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 84, March 1987, WASHINGTON US pages 1268 - 1271 Y. G. YEUNG ET AL 'Purification of the colony stimulating factor 1 receptor and demonstration of its tyrosine kinase activity' see the whole document ---	34

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	JOURNAL OF MOLECULAR BIOLOGY vol. 205, no. 4, 1989, pages 783 - 785 J. M. LA LONDE ET AL 'Crystallization and preliminary X-ray studies of recombinant human granulocyte - macrophage colony stimulating factor' see the whole document ---	1-24
A	SCIENCE vol. 254, 8 November 1991, LANCASTER, PA US page 821825 B. C. CUNNINGHAM ET AL 'Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule' see the whole document -----	25-33,35

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9305548
SA 76062

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

12/10/93

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		EP-A- 0476022	25-03-92
		EP-A- 0561429	22-09-93
		JP-T- 4502014	09-04-92
		WO-A- 9015151	13-12-90
WO-A-9221029	26-11-92	None	
EP-A-0344796	06-12-89	JP-A- 2209895	21-08-90