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(21) International Application Number: PCT/US91/06052 (22) International Filing Date: 23 August 1991 (23.08.91) (30) Priority data: 572,149 23 August 1990 (23.08.90) US (71) Applicant: CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). (72) Inventors: RALPH, Peter ; 119 Crest View Drive, Orinda, CA 94563 (US). CHONG, Kong, T. ; 4343 Fellows Street, Union City, CA 94587 (US). DEVLIN, James ; 1146 Upper Happy Valley Road, Lafayette, CA 94549 (US). ZIMMERMAN, Robert ; 64 La Cresta Road, Orinda, CA 94563 (US). AUKERMAN, Sharon, Lea ; P.O. Box 20852, Oakland, CA 94620 (US). RING, Dav- id, B. ; 1248 Truman Street, Redwood City, CA 94061 (US). MA, Sylvia, Hsieh ; 33535 Stephano Court, Fre- mont, CA 94555 (US).		(74) Agent: McGARRIGLE, Philip, L., Jr.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US). (81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (Euro- pean patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: USES OF RECOMBINANT COLONY STIMULATING FACTOR-1 (57) Abstract A colony stimulating factor, CSF-1, is a lymphokine useful in treating or preventing bacterial, viral or fungal infections, neoplasms, leukopenia, wounds, and in overcoming the immunosuppression induced by chemotherapy or resulting from other causes. CSF-1 is obtained in usable amounts by recombinant methods, including cloning and expression of the murine and human DNA sequences encoding this protein.		

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USES OF RECOMBINANT COLONY STIMULATING FACTOR-1

The present invention relates to the various therapeutic uses of recombinantly produced human colony stimulating factor-1 (CSF-1).

5 The ability of certain factors produced in very low concentration in a variety of tissues to stimulate the growth and development of bone marrow progenitor cells into macrophages and/or granulocytes has been known for nearly 15 years. The presence of such factors in sera, urine samples, and tissue extracts from a number of species is demonstrable using an *in vitro* assay which measures the stimulation of colony
10 formation by bone marrow cells plated in semi-solid culture medium. There are no acceptable *in vivo* assays. Because these factors induce the formation of such colonies, the factors collectively have been called Colony Stimulating Factors (CSF).

More recently, it has been shown that there are at least four subclasses of human CSF proteins which can be defined according to the types of cells found in the
15 resultant colonies. One subclass, CSF-1, results in colonies containing predominantly macrophages. Other subclasses produce colonies which contain both neutrophilic granulocytes and macrophages (GM-CSF); which contain predominantly neutrophilic granulocytes (G-CSF); and which contain neutrophilic and eosinophilic granulocytes, macrophages, and other myeloid cell types (basophils, erythrocytes, and
20 megakaryocytes) (IL-3).

GM-CSF is described by Gough, *et al.*, Nature (1984) 309:763-767. This protein is further described in WO 87/02060, published April 9, 1987, as being useful to treat cancer patients to regenerate leukocytes after traditional cancer treatment, and to reduce the likelihood of viral, bacterial, fungal and parasitic infection in
25 immunocompromised individuals, such as those having acquired immune deficiency syndrome (AIDS). Human IL-3 has been cloned by Yank, Y.C., *et al.*, Cell (1986) 47:3.

There are murine factors analogous to the above human CSFs, including a murine factor called IL-3 which induces colonies from murine bone marrow cells which
30 contain all these cell types plus megakaryocytes, erythrocytes, and mast cells, in various combinations. Murine IL-3 has been cloned by Fung, M.C., *et al.*, Nature (1984) 307:233. See also Yokota, T., *et al.*, Proc Natl Acad Sci (USA) (1984) 81:1070-1074; Wong, G.G., *et al.*, Science (1985) 228:810-815; Lee, F., *et al.*, Proc Natl Acad Sci (USA) (1985) 82:4360-4364; and Cantrell, M.A., *et al.*, Proc Natl Acad Sci (USA) (1985) 82:6250-6254.) These CSFs and others have been reviewed by
35 Dexter, T.M., Nature (1984) 309:746, and Vadas, M.A., *et al.*, J. Immunol. (1983)

130:793, Clark, S.C., Science (1987) 236:1229, and Sachs, L., Science (1987) 238:1374.

The cloning and expression of G-CSF is described in U.S. Patent No. 4,810,643 and a method to purify G-CSF from human oral cancer tissue is described in
5 U.S. Patent No. 4,833,127.

The invention herein is concerned with the recombinant production of proteins which are members of the first of these subclasses, CSF-1. This subclass has been further characterized and delineated by specific radioimmunoassays and radioreceptor assays to suppress specifically CSF-1 activity, without affecting the biological activities
10 of the other subclasses, and macrophage cell line J774 contains receptors which bind CSF-1 specifically. A description of these assays was published by Das, S.K., et al., Blood (1981) 58:630.

A significant difficulty in putting CSF proteins in general, and CSF-1 in particular, to any useful function has been their unavailability in distinct and
15 characterizable form in sufficient amounts to make their employment in therapeutic use practical or even possible. The present invention remedies these deficiencies by providing purified human and murine CSF-1 in useful amounts through recombinant techniques and discloses various therapeutic uses thereof.

Treatment of patients suffering from AIDS with CSF-1, alone or together with
20 erythropoietin and/or an antiviral agent and/or IL-2 is reported in W087/03204, published June 4, 1987. U.S. Patent No. 4,482,485, issued November 13, 1984, states that CSF isolated from human urine can be used for a supporting role in the treatment of cancer. In addition, EP 118,915, published September 19, 1984, reports production of CSF for preventing and treating granulocytopenia and
25 macrophagocytopenia in patients receiving cancer therapy, for preventing infections, and for treating patients with implanted bone marrow.

In addition, CSF-1 is reported to stimulate nonspecific tumoricidal activity (Ralph et al., Immunobiol (1986) 172:194-204). Ralph et al., Cell Immunol (1983) 76:10-21 reported that CSF-1 has no immediate direct role in activation of macrophages
30 for tumoricidal and microbiocidal activities against fibrosarcoma 1023, lymphoma 18-8, and L. tropica amastigotes. Ralph et al., Cell Immunol (1987) 105:270-279 reports the delayed tumoricidal effect of CSF-1 alone and the added tumoricidal effect of a combination of CSF-1 and lymphokine on murine sarcoma TU5 targets. Copending, commonly owned U.S. Application Serial No. 126,221, filed February
35 19, 1988, discloses the synergistic effect of CSF-1 and G-CSF to stimulate the immune system.

In addition, Warren *et al.*, *J. of Immunol.* (1986) 137:2281-2285 discloses that CSF-1 stimulates monocyte production of interferon, TNF and colony stimulating activity. Lee *et al.*, *J. of Immunol.* (1987) 138:3019-3022 discloses CSF-1-induced resistance to viral infection in murine macrophages.

5 In one aspect of the invention, therapeutic treatments based on the ability of CSF-1 to induce resistance to a number of infectious diseases in mammals, including those caused by bacterial, viral or fungal agents, are disclosed. Yet a further aspect concerns the ability of CSF-1 to promote the repair of tissue damage for use in wound healing. Lastly, the invention provides methods of treating tumor cells in mammals by
10 using an effective amount of CSF-1 to treat tumor burden. In one aspect of this indication, the invention relates to methods of enhancing the stimulation of antibody-dependent targeted cellular cytotoxicity using human effector cells, such as, bone marrow derived cells, tissue macrophages, or peripheral blood mononuclear cells against tumor cells, all of which are mediated by a bifunctional antibody. In addition,
15 the invention relates to pharmaceutical compositions comprising CSF-1 or a mixture thereof with a cytokine, lymphokine, or with an excipient for use in the various aforementioned indications.

Figure 1 shows a comparison of the activities of CSF-1 and other colony stimulating factors in enhancing the ability of macrophages to kill tumor cells.

20 Figure 2 shows the ability of the heteroconjugate antibody 113F1 F(ab')₂-3G8 F(ab')₂ at 1 µg/ml to mediate lysis of cancer cells by adherent blood mononuclear cells (AMC) in the presence and in the absence of CSF-1.

Figure 3 shows the ability of the bispecific antibody 2B1 to mediate lysis by AMC preincubated with 14-100 ng/ml of CSF-1, as compared to antibody alone.

25 Figure 4 shows the results of CSF-1 dose range studies on AMC using 100 ng/ml of 2B1.

Figure 5 shows a protective effect of CSF-1 against a lethal dose of murine cytomegalovirus (mCMV).

Figure 6 shows wound closure data in control and CSF-1 treated mice.

30 "Colony stimulating factor-1 (CSF-1 or M-CSF)" refers to those proteins which exhibit the spectrum of activity understood in the art for CSF-1, i.e., when applied to the standard *in vitro* colony stimulating assay of Metcalf, D., *J. Cell Physiol.* (1970) 76:89, it is capable of stimulating the formation of primarily macrophage colonies. Native CSF-1 is a glycosylated dimer; dimerization is reported to be necessary for
35 activity as the monomer is not active in the Metcalf colony stimulating assay (*supra*) or various other *in vitro* bioactivity assays (Ralph, P. *et al.*, *Blood* (1986) 68:633; Stanley, E.R., *et al.*, *J Biol Chem* (1977) 252:4305). As used herein the number of

CSF-1 units corresponds to the number of colonies in the mouse bone marrow colony assay in the titratable range (described in Ralph *et al.*, Blood, *supra*). Contemplated within the scope of the invention and within the definition of CSF-1 are both the dimeric and monomeric forms. The monomeric form may be converted to the dimeric form by *in vitro* provision of suitable refolding conditions as described in copending PCT WO 88/08003, published October 20, 1988, and in U.S. Patent No. 4,929,700, issued May 29, 1990, and the monomer is per se useful as an antigen to produce anti-CSF-1 antibodies.

There appears to be some species specificity: Human CSF-1 is operative both on human and on murine bone marrow cells; murine CSF-1 does not show activity with human cells. Therefore, "human" CSF-1 should be positive in the specific murine radioreceptor assay of Das, 1981, *supra*, although there is not necessarily a complete correlation. The biological activity of the protein will generally also be inhibited by neutralizing antiserum to human urinary CSF-1 (Das, 1981, *supra*). However, in certain special circumstances (such as, for example, where a particular antibody preparation may recognize a CSF-1 epitope not essential for biological function, and which epitope is not present in the particular CSF-1 mutein being tested) this criterion may not be met.

Certain other properties of CSF-1 have been recognized more recently, including the ability of this protein to stimulate the secretion of series E prostaglandins, interleukin-1, and interferon from mature macrophages (Moore, R., *et al.*, Science (1984) 223:178). The mechanism for these latter activities is not presently understood, and for purposes of definition herein, the criterion for fulfillment of the definition resides in the ability to stimulate the formation of monocyte/macrophage colonies using bone marrow cells from the appropriate species as starting materials, and under most circumstances (see above), show inhibition of this activity by neutralizing antiserum against purified human urinary CSF-1, and, where appropriate for species type, exhibit a positive response in the radioreceptor assay. (It is known that the proliferative effect of CSF-1 is restricted to cells of mononuclear phagocytic lineage (Stanley, E.R., The Lymphokines (1981), Stewart, W.E., II, *et al.*, ed., Humana Press, Clifton, NJ), pp. 102-132) and that receptors for CSF-1 are found on cells of this lineage (Byrne, P.V., *et al.*, Cell Biol (1981) 91:848), placental trophoblasts and some other cells).

"Effective amount" signifies an amount effective to perform the function specified, such as to kill tumors or reduce tumor burden or prevent or cure infectious diseases.

"Therapeutic treatment" indicates treating a subject after a disease is contracted, and includes prophylactic therapy.

"Mammals" indicates any mammalian species, and includes rabbits, mice, dogs, cats, primates and humans, preferably humans.

"Immunosuppression" means prevention or diminution of the immune response due to infections, chemical agents, burns, major trauma, or irradiation. Individuals
5 who are immunocompromised are also immunosuppressed.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. In order to effect transformation, the expression system may be included on a vector; however, the
10 relevant DNA may then also be integrated into the host chromosome.

As used herein "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely
15 identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny which have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby specifically incorporated by reference.

20 CSF-1 apparently occurs in numerous forms all of which are included in the embodiments of the present invention. Human CSF-1 cDNA clones coding for CSF-1 pre-pro-polypeptides of three different lengths (256 amino acids; 554 amino acids; and 438 amino acids) have been isolated from cells expressing the single CSF-1 gene (see commonly owned U.S. Patent No. 4,847,201 issued July 11, 1989 and U.S. Patent
25 No. 4,868,119, issued September 19, 1989; Wong, G.G., et al., Science (1987) 235:1504, Kawasaki, et al., Science (1985) 230:291; Ladner et al., Embo J (1987) 6:2693; Cerretti, D.P. et al., Molecular Immunol (1988) 25:761. The CSF-1 proteins useful in the therapies disclosed herein may also be processed by proteolysis including, for example, in the long form, at the Lys residue at 238, the Arg residue at 249, and the
30 Arg residue at 411. It is believed that CSF-1 may occur in nature in one or more C-terminally deleted forms. In addition, CSF-1 proteins lacking the first two or four amino acids have been isolated in active form from the supernatant of the human cell line AGR-ON (equivalent to CEM-ON; ATCC No. CRL-8199; Takahashi, M., et al., Biochem Biophys Res Comm (1988) 152:1401 and U.S. Patent No. 4,675,291 issued
35 June 23, 1987). CSF-1 protein comprising monomers ending at amino acid 145 are reported to have in vitro biological activity (European Patent (EP) Publication No. 261,592 published March 30, 1988). Some biological activity is reported for a dimeric

CSF-1 protein composed of monomers ending at amino acid 132 (EP 328,061 published August 16, 1989). The monomeric CSF-1 polypeptide (whether clipped at the C-terminus or not) may also refold to form multimers, most frequently dimers.

Native human urinary CSF-1 has been isolated as a highly glycosylated dimer of 45-90 kD, depending on the source, method of measurement and identity of the reporter. The recombinantly produced unglycosylated CSF-1 reported by Wong, *et al.*, (*supra*) appears to have a monomeric molecular weight of approximately 21 kD. On the other hand, the molecular weight calculated on the basis of the amino acid sequence deduced for the "short" 224 amino acid form of CSF (SCSF) by Kawasaki *et al.*, (*supra*) (see also U.S. Patent No. 4,847,201 (*supra*) and commonly owned PCT Publication No. WO86/04607 published August 14, 1986) is on the order of 26 kD, while that of the "long" 522 amino acid form (LCSF) is calculated to be on the order of 55 kD (Wong, *et al.*, (*supra*); Ladner *et al.*, (*supra*); commonly owned EP 272,779, published June 29, 1988; and U.S. Patent No. 4,868,119 corresponding to PCT Publication No. WO87/06954, published November 19, 1987). When deleted constructs of these genes are expressed in *E. coli* (where glycosylation does not occur), they, of course, give rise to proteins of considerably lower molecular weight.

Other forms of CSF-1 proteins useful in the present invention include the polymer conjugated CSF-1 described in commonly owned U.S. Patent No. 4,847,325. The transmembrane region deletion mutants disclosed in EP 249,477 published December 16, 1987 and the glycosylation site deletion mutants disclosed in EP 272,779, *supra*, are also considered to be useful in the presently disclosed therapies.

Methods for the production of these various forms of CSF-1 from various sources are reported in U.S. Patent No. 4,879,227 issued November 7, 1989; WO 86/04587 published August 14, 1986; WO 89/10407 published November 2, 1989; WO 88/08003; *supra* and EP 276,551 published August 3, 1988.

It is, of course, well known that bacterially produced mature proteins which are immediately preceded by an ATG start codon may or may not include the N-terminal methionine, and it is shown in EP 272,779 (*supra*) that deletion of residues 1 and 2 (both glutamic acid) or residues 1-3 (Glu-Glu-Val) aids in this manner. Deletions are noted by a ▼ followed by the number of amino acids deleted from the N-terminal sequence, or by the number of amino acids remaining when residues are deleted from the C-terminal sequence. Thus, the N-terminal deletions referred to above having the first 2 and the first 3 residues deleted are designated N▼2 and N▼3, respectively. C-terminal truncations of CSF-1 resulting in proteins of 150, 158, 190 and 221 amino acids in length for example are referred to as C▼150, C▼158, C▼190 and C▼221, respectively. A 221 amino acid CSF-1 molecule derived from LCSF having an

N-terminal deletion of 3 amino acids is denoted by LCSF/N▼3 C▼221, for example. Amino acid substitutions are designated by reference to the position of the amino acid which is replaced. For example, substitution of the cysteine residue at position 157 in Figure 4 of Ladner *et al.*, (*supra*) by serine is referred to as CSF-1 Ser₁₅₇.

5 In summary, in addition to the N-terminal and C-terminal deletions and aggregations, individual amino acid residues in the chain may be modified by oxidation, reduction, deletion or other derivatization, and these proteins may also be cleaved and/or polymerized to obtain dimeric products that retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the
10 definition, and are specifically included as substantial equivalents. CSF-1 derived from other species may fit the definition of a protein having activity of "human CSF-1" by virtue of its display of the requisite pattern of activity as set forth above with regard to human substrate.

 As is the case for all proteins, the precise chemical structure depends on a
15 number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular protein may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their activity when placed in suitable environmental conditions are included in the definition. Further, the primary amino acid sequence may be augmented by derivatization using sugar moieties (glycosylation)
20 or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like, more commonly by conjugation with saccharides, polyethylene glycols (PEGs), and polyoxyethylene glycols (POGs) as shown in U.S. Patent No. 4,847,325. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced *in vitro*. In
25 any event, such modifications are included in the definition so long as the activity of the dimeric protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in the various assays.

 Further, individual amino acid residues in the chain may be modified by
30 oxidation, reduction, or other derivatization, and the protein may be cleaved to obtain fragments which retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the definition.

 Modifications to the primary structure itself by deletion, addition, or alteration
of the amino acids incorporated into the sequence during translation can be made
35 without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence which falls within the definition of proteins "having an amino acid sequence substantially equivalent to that of CSF-1."

Indeed, human- and murine-derived CSF-1 proteins have nonidentical but similar primary amino acid sequences which display a high homology.

The CSF-1 proteins of the invention are capable both of stimulating monocyte-precursor/macrophage cell production from progenitor marrow cells, thus enhancing the effectiveness of the immune system, and of stimulating such functions of these differentiated cells as the secretion of lymphokines in the mature macrophages.

In one application, these proteins are useful as adjuncts to chemotherapy. It is well understood that chemotherapeutic treatment results in suppression of the immune system. Often, although successful in destroying the tumor cells against which they are directed, chemotherapeutic treatments result in the death of the subject due to this side effect of these toxic agents on the bone marrow cells. Administration of CSF-1 to such patients, because of the ability of CSF-1 to mediate and enhance the growth and differentiation of bone marrow-derived precursors into macrophages and monocytes and to stimulate the function of these mature cells, results in a re-stimulation of the immune system to prevent this side effect, and thus to prevent the propensity of the patient to succumb to secondary infection.

CSF-1 may also be used to cure leukopenia, a disease involving a deficiency in the total number of white blood cells. Neutropenia reflects a deficiency affecting principally the polymorphonuclear leukocytes (neutrophils, granulocytes) and may be due to various infections, certain drugs (e.g., cytotoxic drugs) or ionizing radiations. Thus, in vivo administration of CSF-1 can be used to induce stem cells to indirectly increase the production of polymorphonuclear leukocytes, thereby increasing the count of white blood cells.

Other patients who would be helped by such treatment include those being treated for leukemia through bone marrow transplants; they are often in an immunosuppressed state to prevent rejection. For these patients also, the immunosuppression could be reversed by administration of CSF-1.

In general, any subject suffering from immunosuppression whether due to chemotherapy, bone marrow transplantation, or other, forms of immunosuppression such as disease (e.g., acquired immune deficiency syndrome) would benefit from the availability of CSF-1 for pharmacological use.

Opportunistic infections can be contracted as a result of immunosuppression. For example, AIDS related opportunistic infections are described in Mills *et al.*, 1990, Scientific American 263:51-57, which is hereby incorporated by reference in its entirety. Mills *et al.* show that common opportunistic infections are caused by the following agents: Cytomegalovirus, Pneumocystis carinii, Candida albicans, Varicella-Zoster virus, Epstein-Barr virus, Toxoplasma gondii, Mycobacterium avium,

Cryptococcus neoformans, etc. The authors also describe various agents that are used to treat these infections. It is contemplated that CSF-1 can be combined with one or more of those agents to treat these and other opportunistic infections.

Additionally, it is envisioned that CSF-1 is used to treat Cryptococcal infections, such as cryptococcal meningitis. Cryptococcal meningitis is the most common form of fungal meningitis in the United States and the third most important agent of neurologic disease after Human Immunodeficiency Virus (HIV) and Toxoplasma gondii in AIDS patients. Cryptococcosis will develop at some point during their illness in 5 to 7% of patients with AIDS in the U.S. while disseminated cryptococcosis may develop in up to a third of African AIDS patients.

The therapeutic treatment for these opportunistic infections can be similar to that described below for fungal diseases. For example, CSF-1 can be administered at similar dose levels and on the same schedule.

In addition, subjects could be supplied enhanced amounts of previously differentiated macrophages to supplement those of the indigenous system, which macrophages are produced by in vitro culture of bone marrow or from blood monocytes, or other suitable preparations followed by treatment with CSF-1. These preparations include those of the patient's own blood monocytes or bone marrow derived cells, which can be so cultured and returned for local or systemic therapy.

The ability of CSF-1 to stimulate production of lymphokines by macrophages and to enhance their ability to kill target cells also makes CSF-1 directly useful in treatment of neoplasms and infections. Moreover, treatment of wounds with CSF-1 will promote tissue repair.

CSF-1 stimulates the production of interferons by murine-derived macrophages (Fleit, H.B., et al., J. Cell Physiol. (1981) 108:347), and human, partially purified, CSF-1 from MIAPaCa cells stimulates the poly(I):poly(C)- induced production of interferon and TNF from human monocytes as illustrated in PCT publication WO86/04607, supra. In addition, CSF-1 stimulates the production of myeloid CSF by human blood monocytes.

Moreover, for the various uses described herein, CSF-1 can be employed in conjunction with other efficacious agents, including antibodies; lymphokines; cytokines; or macrophage activating agents, such as, e.g., IFN-alpha, IFN-beta, IFN-gamma, IL-2, TNF; or muramyl dipeptide and analogs thereof to treat tumors.

Also illustrated below is a demonstration of the ability of CSF-1 (from murine L-cell-conditioned medium and E. coli produced human recombinant CSF-1) to stimulate normal C3H/HeN mouse peritoneal macrophages to kill murine sarcoma TU5 targets. This activity is most effective when the CSF-1 is used as pretreatment and

during the effector phase. The ability of CSF-1 to do so is much greater than that exhibited by other colony stimulating factors.

CSF-1 may also be employed to augment the lymphokine-induced antibody-dependent cellular cytotoxicity (ADCC) or targeted ADCC by macrophages or natural killer cells against tumor cells. This activity is particularly effective when CSF-1 is used in combination with IL-2, IFN-alpha, IFN-beta or IFN-gamma.

The ability of CSF-1 to enhance targeted ADCC activity is believed to be dependent on the kind of the antibody, the dose of CSF-1, and the effector to target ratio. Targeted cellular cytotoxicity is thought to rely on cell surface receptors on cytotoxic cells, such as monocytes, macrophages, natural killers, etc. It is known that the expression of one of these cell surface receptors, CD16, is enhanced by culturing these effector cells in CSF-1, with or without additional lymphokines, such as IL-2. If the cytotoxic cell is positioned up against the target cell, cytotoxicity is enhanced. A bifunctional antibody can promote this process by binding to a target cell through one of its combining sites, and to the lysis promoting receptor on the cytotoxic cell through its second combining site, thereby joining the two cell types and causing the cytotoxic cell to deliver a kill signal. "Bifunctional antibodies" include those produced by *in vivo* recombination of antibody chains in trioma or hybrid hybridoma cell lines and those produced by *in vitro* chemical conjugation of two antibodies or antibody fragments; the latter chemically linked antibodies are referred to as heteroconjugates.

In the instant invention, such bifunctional antibodies include either hybrid hybridoma derived bispecific or heteroconjugated antibodies that target the CD16 antigen known as the human Fc receptor III (FcRIII) on leukocytes. 3G8, a murine hybridoma secreting an IgG₁ monoclonal antibody to human FcRIII, is described in Unkeless, J.B., et al., *Ann Rev Imm* (1988) 6:251. The use of this antibody and monoclonal antibody 520C9 to develop a hybrid hybridoma derived bispecific antibody 2B1, is described in co-pending U.S. Serial No. 07/249,710, filed September 27, 1988. The resulting bispecific antibody exhibits binding to the CD16 Fc receptor III positive cells, as well as to breast cancer cells that display the positive proto-oncogene product erbB-2. 3G8 has also been chemically cross-linked (using chemical crosslinkers as described by, for example, Karpovsky, et al., *J. Exp Med* (1984) 160:1686) with 113F1 antibody, a murine monoclonal antibody to a breast cancer associated antigen (U.S. Patent No. 4,753,894), producing an antibody also having bifunctional specificity.

The *in vitro* effective dose of CSF-1 in such a targeted cytotoxic assay, is in the range of 10-200 ng/ml. However, its *in vivo* dosage is dependent upon various factors including the severity of the cancer, host immune status, body weight, the ratio of

effector to target cells, many of which may only be determined on a case-by-case basis. CSF-1 should be administered in a dosage which does not cause a systemic toxic reaction but elicits the potentiating response on the effector cells.

The in vitro effective dosages of the bifunctional antibody are in the range of 1
5 ng/ml to 200 ng/ml. In vivo dosage again depends on a number of factors, including the clinical estimate of tumor size, the extent of metastasis and the biodistribution of the active drugs and of the cells that are activated. The effective in vitro effector to target cell ratio is approximately 10:1 to 80:1. The actual effector to target ratio in vivo depends on the accessibility of the tumors to the effector cells and antibodies.

10 In addition, the ability of murine cells to attack infectious organisms, including viruses such as those from the Herpesvirus genera, for example, cytomegalovirus; bacterial agents including those causing Gram-negative sepsis, and fungi is enhanced by CSF-1. (Murine CSF-1 is inconsistently reported to stimulate murine macrophage to be cytostatic to P815 tumor cells (Wing, E.J., et al., J. Clin. Invest. (1982) 69:270)
15 or not to kill other leukemia targets (Ralph, P, et al., Cell Immunol (1983) 76:10). Nozawa, R.T., et al., Cell Immunol (1980) 53:116, report that a CSF-1 preparation may stimulate macrophage in vitro to ingest and kill Candida.)

Additionally, it has been discovered that CSF-1 is effective in treating fungal infections in humans. These fungal infections typically arise in immunosuppressed
20 patients (for example, patients that have bone marrow transplants, AIDs, etc.), but can also occur in non-immunosuppressed individuals. Fungi from the following genres that can be treated: Candida, Aspergillus, Cryptococcus, Histoplasma, Blastomyces, Coccidioides, Paracoccidioides, Mucor, Rhodotorula, Sporothrix, Dermatophytosis, Pseudallescheria, Prototheca, Rhinosporidium, or fungi that cause mycetoma or
25 chromomycosis, etc. (see Principles and Practice of Infectious Diseases, 3rd Edition, Mandell et al., Churchill Livingstone [1990] pages 1942-2016).

Preferably, CSF-1 is parenterally administered until the fungal infection is cleared as determined by negative culture results. CSF-1 can be administered subcutaneously, by continuous infusion, by bolus injection, or by constant infusion.
30 By "continuous infusion" is meant at least 24 hours of dosing and by "constant infusion" is meant 24 hours or less of dosing. CSF-1 is preferably administered by constant infusion lasting between 1 and 4 hours. The daily CSF-1 dose that is effective to treat fungal infections is preferably between 0.01 to 50 mg/M², more preferably between 0.05 to 10 mg/M², most preferably between 0.5 and 5 mg/M². Preferably,
35 CSF-1 is administered for at least 14 days, more preferably for 21-59 days. Other dose schedules can also be used if the above treatment causes unwanted effects (however,

minimal effects have been observed at doses of up to 30 mg/M²/day by i.v. bolus) or if more efficacious results can be shown.

Preferably, CSF-1 is used to treat Candida and Aspergillus infections as described above and specifically as shown below. Cryptococcus infections such as
5 cryptococcal meningitis can be treated similarly.

Thus, in addition to overcoming immunosuppression per se, CSF-1 can be used to destroy the invading organisms or malignant cells indirectly or directly by stimulation of macrophage secretions and activity. This latter activity may be enhanced by CSF-1 therapy in combination with an antimicrobial agent such as for example, one
10 or more antiviral, antifungal or antibacterial agents.

Examples of antifungal agents include: Amphotericin B, Fluconazole (Diflucan), 5 fluoro-cytosine (Flucytosine, 5-FC), Ketoconazole, Miconazole, Intraconazole, etc. (see also Mandell *et al.* above at pages 361-370 or Mills *et al.* at pages 54-55). As an example of the clinical spectrum of one of the above antifungal
15 agents, Amphotericin typically inhibits the following fungi and protozoa: Aspergillus fumigatus, Paracoccidioides brasiliensis, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, Mucor mucedo, Rhodotorula spp., Sporothrix shenckii, Blastomyces dermatitidis, Candida spp., Leishmania spp., Naegleria spp., and Acanthamoeba spp. The antifungal agents can be administered before, during, or
20 after the CSF-1 is given. Doses of these agents are known to those of ordinary skill in the art and include daily dose ranges such as 0.4 to 0.6 mg/kg/day for Amphotericin B, 200 mg/day to 400 mg/day for Fluconazole, about 150 mg/kg/day for 5-Fluoro-cytosine, and 200 mg/day to 400 mg/day for Ketoconazole. These ranges are merely exemplary and are not to be construed as limiting in any way. Also, it is contemplated that CSF-1
25 can be combined with other agents that are, or will be, effective at treating fungal infections. The antifungal agents can be conjugated to polymers to adjust their *in vivo* half-life and toxicity, see U.S. Serial No. 365,914, which is hereby incorporated by reference in its entirety.

As stated above CSF-1 can be combined with other antimicrobial agents, such
30 as antibacterials. Examples of antibiotics that can be combined with CSF-1 include those selected from the following categories: β -lactam rings (penicillins), amino sugars in glycosidic linkage (aminoglycosides), macrocyclic lactone rings (macrolides), polycyclic derivatives of naphthacene-carboxamide (tetracyclines), nitrobenzene derivatives of dichloroacetic acid, peptides (bacitracin, gramicidin, and polymyxin),
35 large rings with a conjugated double bond system (polyenes), sulfa drugs derived from sulfanilamide (sulfonamides), 5-nitro-2-furanyl groups (nitrofurans), quinolone

- carboxylic acids (i.e., nalidixic acid), and many others. The groups of antibiotics mentioned above are examples of preferred antibiotics, examples of antibiotics within those groups are: peptide antibiotics, such as amphotericin, bacitracin, bleomycin, cactinomycin, capreomycins, colistin, dactinomycin, enduracidin, gramicidin A, gramicidin J(S), mikamycins, polymyxins, stendomycin, thiopeptin, thiostrepton, tyrocidines, viomycin, virginiamycins, and actinomycin (see Encyclopedia of Chemical Technology, 3rd edition, Kirk-Othmer- Editors, Volume 2, at page 991 (1978) which is hereby incorporated by reference in its entirety); aminoglycosides, such as streptomycin, neomycin, paromomycin, gentamycin, ribostamycin, tobramycin, amikacin, and lividomycin (see Kirk-Othmer, Volume 2 at pages 8-19); β -lactams, such as benzylpenicillin, methicillin, oxacillin, hetacillin, piperacillin, amoxicillin, and carbenicillin (see Kirk-Othmer, Volume 2 at page 871); chloramphenicol (see Kirk-Othmer, Volume 2 at page 920); lincosaminides, such as clindamycin, lincomycin celesticetin, desalicytin (see Kirk-Othmer, Volume 2 at page 930); macrolides, such as erythromycin A-E, lankamycin, leucomycins, and picromycin (see Kirk-Othmer, Volume 2 at page 937); nucleosides, such as 5-azacytidine, amicitin, puromycin, and septacidin (see Kirk-Othmer, Volume 2 at page 962); oligosaccharides, such as curamycin, and everninomicin B (see Kirk-Othmer, Volume 2 at page 986); phenazines, such as, myxin, lomofungin, iodinin, etc. (see Kirk-Othmer, Volume 3 at page 1); polyenes, such as amphotericins, candididin, nystatin, etc. (see Kirk-Othmer, Volume 3 at page 21); polyethers (see Kirk-Othmer, Volume 3 at page 47); tetracyclines, such as chlortetracycline, oxytetracycline, demeclocycline, methacycline, doxycycline, and minocycline (see Kirk-Othmer, Volume 3 at page 65); sulfonamides, such as sulfathiazole, sulfadiazine, sulfapyrazine, and sulfanilamide (see Kirk-Othmer, volume 2, page 795); nitrofurans, such as nitrofurazone, furazolidone, nitrofurantoin, furium, nitrovin, and nifuroxime (see Kirk-Othmer, volume 2, page 790); quinolone carboxylic acids, such as nalidixic acid, piromidic acid, pipemidic acid, and oxolinic acid (see Kirk-Othmer, volume 2, page 782).
- CSF-1 can also be combined with antiviral agents, such as amantadine, rimantadine arildone, ribavirin, acyclovir, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (DHPG), vidarabine (ARA-A), ganciclovir, enviroxime, foscarnet, interferons (alpha-, beta- and gamma-), ampligen, podophyllotoxin, 2,3-dideoxycytodine (DDC), iododeoxyuridine (IDU), trifluorothymidine (TFT), dideoxyninosine (ddI), dideoxycytodine (ddC), zidovudine and specific antiviral immune globulins (see Sanford, J.P. Guide to Antimicrobial Therapy, West

Bethesda:Antimicrobial Therapy, Inc., 1989, pp.88-93; and Harrison's Principles of Internal Medicine, 11th Edition, Braunwald, E. et al. Eds. New York:McGraw-Hill Book Co., 1987, pp 668-672).

5 Finally, the CSF-1 may be used to promote the repair of tissues for wound healing when applied either locally or systemically. CSF-1 may recruit macrophages (Wang, J.M., et al., J Immunol (1988) 141:575), as well as induce them to provide connective tissue growth factors such as platelet-derived growth factor (PDGF), and active factors including tumor necrosis factor (TNF), as the stimulus for cell proliferation. Wound macrophages are reported to release substances that stimulate
10 fibroplasia, collagen synthesis, and angiogenesis in vivo (Hunt T.K., et al., Surgery (1984) 96:48).

The CSF-1 of the invention may be formulated in conventional ways standard in the art for the administration of protein substances. Administration by injection is one preferred route; and such formulations include solutions or suspensions,
15 emulsions, or solid composition for reconstitution into injectables or gel formulations. Suitable excipients include, for example, Ringer's solution, Hank's solution, water, saline, glycerol, dextrose or mannitol solutions, and the like. While liquid solutions of CSF-1 may be used directly on or under wound dressings, reconstituted compositions are useful for salves, gel formulations, foams and the like for wound healing.
20 Reconstituted gel formulations provide a controlled delivery system for CSF-1 at a wound site. Controlled release refers to drug release sufficient to maintain a therapeutic level over an extended period of time, such as up to 24 hours or more, preferably in the range of 24-72 hours. Increased contact time of growth factors may be necessary to achieve a significant increase in the rate of wound healing.

25 In addition, the CSF-1 of the invention may be preincubated with preparations of cells in order to stimulate appropriate responses, and either the entire preparation or the supernatant therefrom introduced into the subject. As shown hereinbelow, the materials produced in response to CSF-1 stimulation by various types of blood cells are effective against desired targets, and the properties of these blood cells themselves to
30 attack invading organisms or neoplasms may be enhanced. The subject's own cells may be withdrawn and used in this way, or, for example, monocytes or lymphocytes from another compatible individual employed in the incubation.

As discussed previously and particularly with regard to the subject matter disclosed in U.S. Patent No. 4,847,201 (supra), the complete coding sequences for a
35 number of human CSF-1 proteins are now available, and expression vectors applicable to a variety of host systems have been constructed and the coding sequence expressed. In addition to those expression systems provided in U.S. Patent No. 4,847,201,

expression systems employing insect cells utilizing the control systems provided by baculovirus vectors have been described (Miller, D.W., et al., in Genetic Engineering (1986) Setlow, J.K., et al., eds., Plenum Publishing, Vol. 8, pp. 277-279, U.S. Patent No. 4,745,051, issued May 17, 1988, and copending U.S. Serial No. 077,188, filed July 24, 1987. Insect cell-based expression can be in Spodoptera frugiperda. These systems are also successful in producing CSF-1. Mammalian expression has been accomplished in COS-7, CHO, mouse, and CV-1 cells, and also can be accomplished in COS-A2, hamster, and murine cells.

The variety of hosts available, along with expression vectors suitable for such hosts, permits a choice among post-translational processing systems, and of environmental factors providing conformational regulation of the protein thus produced. Thus, the availability of this information provides CSF-1 proteins in sufficient quantity for application in the various therapies discussed herein.

Among the vectors disclosed in the aforementioned patent publications, the plasmids pLCSF221A (which contains the gene encoding asp₅₉LCSF/N ∇ 3C ∇ 221) and pcCSF-17 (which contains the gene encoding SCSF) are preferred for procaryotic and eukaryotic expression, respectively, of human CSF-1. The plasmid pCSF221A (hereinafter E. coli (221)) transformed into E. coli strain DG116, was deposited with the ATCC on April 14, 1987 under the Accession No. 67390. The plasmid pcCSF-17 in E. coli MM294 was deposited with the ATCC on June 14, 1985 under the Accession No. 53149.

Also preferred are CSF-1 proteins which comprise the amino acid sequences containing the first 3-150 or 4-150 amino acids of SCSF and LCSF and the C-terminal deletions, such as LCSF/ ∇ 221.

The activity of CSF-1 was determined in the following examples using partially purified MIAPaCa CSF-1, murine L cell CSF-1, CV-1-produced recombinant material or E. coli-produced human CSF-1. CSF-1 was shown to enhance the production of interferon (IFN) and tumor necrosis factor (TNF) by induced human monocytes by up to 10 to 30-fold. CSF-1 also was demonstrated to stimulate macrophage antitumor toxicity in vitro, to inhibit tumor growth in vivo, to protect mice from lethal bacterial infection, to promote the repair of tissue damage in vivo, to inhibit the growth of cytomegalovirus in vivo, and to inhibit the growth of yeast in vivo.

The following examples are illustrative, not limiting, of the therapeutic uses claimed herein.

Stimulation of TNF Production by Human Monocytes

MIAPaCa CSF-1 was purified from the supernatant by calcium phosphate gel filtration and lentil lectin chromatography. For assay of lymphokine production, peripheral blood-adherent cells were incubated in duplicate flasks containing 10^7 cells each. One flask was treated with 1000 U/ml CSF-1 purified as above. After 3 days, the cells were harvested, and washed, and resuspended at a cell concentration of 5×10^5 /ml and plated in 24-well plates at 0.5 ml/well. The wells were treated with 10 μ g/ml lipopolysaccharide (LPS) and 20 ng/ml PMA for 48 hr and the supernatants were harvested for TNF assay. Cells treated with CSF showed TNF secretions approximately 9-fold higher than the untreated cells (1500 U/ml, compared to 162 U/ml).

Stimulation of Interferon Production by Human Monocytes

In an analogous experiment to determine the effect of CSF-1 on interferon production, peripheral blood-adherent cells were incubated for 3 days in the presence and absence of 1000 U/ml CSF-1, as described above, harvested, resuspended at 5×10^5 /ml, and plated in a 24-well plate, as described above. The cells were induced for interferon production by addition of varying amounts of poly(I):poly(C). The supernatants were assayed for interferon production by their cytopathic effect on VSV-infected GM 2504 cells. The CSF-1-stimulated cells showed production of 100 U/ml when induced with 50 μ g/ml poly(I):poly(C), whereas comparably induced untreated cells produced less than 3 U/ml.

Stimulation of Myeloid CSF Production by Human Monocytes

Monocytes were incubated $\pm$ CSF-1 for 3 days and then induced for production of myeloid CSF as in Table 1. The three representative experiments shown used blood from different donors.

17

Table 1
Myeloid CSF (U/ml)

5	Induction	Exp. 1		Exp. 2		Exp. 3	
		-CSF	+CSF	-CSF	+CSF	-CSF	+CSF
10	medium	0	0	0	0	0	0
	0.1 µg/ml 80+17 LPS	-	-	0	0	0	
15	1 µg/ml LPS 377±57	0	700±72	40±20	200±20	103±12	
20	0.1 µg/ml 1280±60 LPS + 2 ng/ml PMA	-	-	617±50	993±101	1120±82	
25	1 µg/ml LPS 1080±122 + 2 ng/ml PMA	283±42	983±252	360±92	1400±180	537±47	
30	2 ng/ml PMA 716±76	-	370±17	297±6	183±15	380±52	

Therefore, CSF-1 stimulates myeloid CSF or colony stimulating activity production.

Stimulation of Tumor Cell Killing by Murine Macrophages:

35

Comparison to other Colony Stimulating Factors

To assay macrophage stimulation, murine CSF-1 obtained from L-cell-conditioned medium, was used as a model for the recombinantly produced CSF-1 from pcCSF-17 in an assay which showed stimulation of the ability of murine macrophages to kill sarcoma targets. In this assay, 2 hr adherent C3H/HeN mouse

40 peritoneal macrophages were incubated for 1 day *in vitro* with and without CSF-1 and then mixed at a 20:1 ratio with ³H-thymidine-labeled mouse sarcoma TU5 cells along with 10% (v/v) ConA-induced (10 µg/ml) spleen lymphokine (LK), which contains gamma interferon. The LK preparation can be replaced by purified gamma interferon in this assay. The release of labeled thymidine over the following 48 hours was used as a

45 measure of tumor cell killing. The effect of adding CSF-1 as murine L-cell-conditioned medium containing 1200 U/ml CSF-1 is shown in Table 2.

Purified murine CSF-1 and rhCSF-1 from CV-1 and *E. coli* (221) have also been effective in this assay.

Table 2

5	Treatment		Kill %	Increase Due to CSF-1 %
	DAY 1	DAY 13		
10	--	--	13	--
	--	LK	39	--
	--	CSF-1+LK	49	26
	CSF-1	LK	51	31
	CSF-1	CSF-1+LK	60	54
15	--	--	3	--
	--	LK	35	--
	--	CSF-1+LK	47	34
	CSF-1	--	7	--
	CSF-1	LK	49	40
20	CSF-1	CSF-1+LK	69	97

Increase in the ability to kill the target cells was noted whether CSF-1 was added during the preliminary 1 day of growth or during the period of induction; however, the most dramatic effects were observed when CSF-1 was present during both of these periods.

25 The possibility of contaminating bacterial LPS as the cause of stimulation of monocytes and macrophages was excluded: The LPS content of the applied CSF-1 was low (<0.3 ng/3000 U CSF-1, by Limulus amoebocyte lysate (LAL) assay); activity was removed by application to an anti-CSF-1 column; polymyxin B was used to neutralize LPS; the macrophages from C3H/HeJ mice respond to CSF-1 but not to
30 LPS.

Effect of Other Myeloid CSFs

CSF-GM was prepared from 6 mouse lungs obtained 5 hours after intravenous (i.v.) administration of 5 µg LPS. The lungs were chopped and incubated for 3 days in serum free medium, and the supernatant was depleted of CSF-1 using a YYG106
35 immunoaffinity column as described in WO 86/04587, *supra*, (CSF-1 content reduced from 270 U/ml to 78 U/ml). CSF-G was prepared from similarly treated LD1 (melanoma cell line) serum free medium. Both CSF-GM and CSF-G contents were assayed at 2000 U/ml by colony stimulating assay.

40 The peritoneal macrophages were incubated with 40% of either of the foregoing media or with L-cell medium assayed at 2000 U/ml CSF-1 for 1 day, and then incubated for 48 hours either with additional medium or with LK, and assayed for TU5 killing as described above.

The results are shown in Figure 1. While CSF-1 showed marked enhancement of toxicity to TU5, neither CSF-G nor CSF-GM had any effect.

In Vitro Test of CSF-1 as Stimulator of ADCC

5 CSF-1 purified from M1APaCa cell line (approximately 40% purity, specific activity approximately 2×10^7 U/mg), murine L-cell conditioned medium (specific activity approximately 2.3×10^5 U/mg), and recombinant human (rh) from CV-1 (>95% purity, specific activity approximately 4×10^7 were found to stimulate mouse macrophage ADCC to tumor targets in combination with IL-2 or alpha-, beta- or
10 gamma-IFN.

In the ADCC assay, female C3H/HeN or C3H/HeJ mice were injected intraperitoneally (i.p.) with 1.5 ml proteose peptone (Difco Laboratories, Detroit, MI). After 3 days, the peritoneal exudate cells at 3×10^5 big cells/0.5 ml alpha-MEM medium plus 10% heat-inactivated fetal calf serum were adhered in replicate 1 ml wells
15 in parallel sets. After 2 hours, the wells were washed thoroughly 3 times with PBS, and CSF-1 or lymphokine was added and incubated for 2 days at 37°C. The cell population was >95% macrophage by morphology and cell numbers recovered in parallel wells at day 2 were similar for the different treatments. On day 2, heat-inactivated antiserum (anti-Thy-1, rabbit anti-mouse brain, Accurate Chemicals,
20 Westbury, NY) was added to one of the parallel sets at various dilutions. The target, R1.1, a T-lymphoma cell line, was added to macrophage wells and to parallel wells without macrophages $\pm$ CSF-1, lymphokine, or antiserum.

Since high concentrations (1 μ g/ml) of bacterial LPS stimulate macrophage ADCC, the murine and human CSF-1 preparations were tested using the LAL assay
25 and had less than 0.2 ng/ml LPS.

The macrophages were then tested at 3:1 effector:target ratio for ADCC by introducing 10^5 R1.1 targets plus or minus antiserum and counting live target cells at 9, 24, 48 and 96 hr. Growth of R1.1 with control macrophages plus antibody was the same as that with control or cytokine-treated macrophages in the absence of antibody or
30 that of R1.1 alone $\pm$ antibody $\pm$ cytokines. The results are shown in Table 3.

Table 3

Macrophage Treatment	Percent ADCC* Kill with Time		
	9 hours	24 hours	48 hours
Medium	0	0	0
M-CSF	0	0	0
IFN-gamma	44±5	63±3	72±3
M-CSF (1000U) + IFN-gamma	88±1	>96	98±1
M-CSF (100U) + IFN-gamma	83±6	93±3	92±5

* %ADCC kill = 100 (y-x)/y where y = target cell number minus antiserum and x = target cell number plus 1:20,000 dilution antiserum. IFN-gamma was used at 5 U/ml.

IFN-alpha and IFN-beta at 50 U/ml had about the same ADCC-stimulation effect as 5 U/ml IFN-gamma. IFN-alpha and IFN-beta at 5 U/ml had essentially no effect on ADCC, but in the presence of CSF-1 stimulated tumor killing to levels seen using 50 U/ml of either IFN alone. Similar effects were seen with rhIL-2: treatment of macrophages for two days with 5 U/ml IL-2 alone significantly boosted macrophage ADCC. CSF-1 moderately enhanced this strong IL-2 induced activity. However, when IL-2 was used at lower, ineffective concentrations of 1 U/ml or 0.2 U/ml, the addition of CSF-1 showed a strong enhancing effect on tumoricidal activity.

Other lymphokines were tested as primary stimulators of ADCC. Incubation of macrophages for two days with rhTNF at 1, 10 or 100 U/ml alone or with 1000 U/ml CSF-1 did not significantly induce ADCC activity. rhIL-1 alpha or beta at 0.2 to 50 U/ml and murine rIL-4 at 1 to 100 U/ml also did not stimulate ADCC alone or with CSF-1. Attempts were made to find other cofactors which could substitute for CSF-1. Murine rGM-CSF and rIL-3 tested at 10, 100 or 1000 U/ml did not boost ADCC alone or with IFN-gamma in the standard two-day pretreatment of macrophages, in contrast to CSF-1. These cytokines, after incubation 2 days in medium, had no effect on growth of RL1 targets in the absence of macrophages.

In Vitro Targeted ADCC Assays

The effects of CSF-1 on cultured human peripheral blood monocytes were studied to determine whether pretreatment with CSF-1 could enhance targeted ADCC.

Human effector cells were isolated from donor buffy coats obtained from Stanford University Blood Bank (Palo Alto, CA). Mononuclear cells were separated

by Ficoll-Hypaque differential centrifugation. To isolate adherent mononuclear cells (AMC), total mononuclear cells were plated into 24-well tissue culture plates and allowed to adhere for 30 minutes at 37°C, 5% CO₂. Nonadherent cells were washed off with warm Hank's balanced salt solution with 50 µg/ml gentamicin. The viabilities of all effector cell preparations were >95% by trypan blue dye exclusion. Adherent mononuclear cells were stained with FITC-anti LeuM3 (Becton Dickinson, Mountain View, CA) and analyzed on an EPICS V cell sorter to be >85% LeuM3 positive.

The antibody heteroconjugate 113F1 F(ab')₂-3G8 F(ab')₂, a chemically linked antibody recognizing both a breast cancer associated antigen and the human FcRIII (CD16), and the hybrid hybridoma derived bispecific antibody 2B1, which has anti-CD16 and anti-erbB-2 activities, were used in the following experiments. Both of these antibodies mediate good specific lysis of SK-Br-3 tumor target cells using human total mononuclear cells as effectors.

One day before an ADCC assay, target cells in T75 flasks (50% confluent) were labeled with 62.5 µCi of ³H thymidine (New England Nuclear, 6.7 µCi/mmol) in 25 ml medium. After 30 hours, the cells were trypsinized off the flasks and washed 3 times. Forty thousand labeled target cells were used per well in the assays.

Medium used throughout the assays for diluting effector cells, antibodies and CSF-1 was AIM.V serum free medium (Gibco) with 8 mM glutamine. Final total volume was 1 ml/well. For CSF-1 treatment, medium with or without CSF-1 was added to effector cells and incubated for 2-3 days. Antibodies and labeled target cells were then added. Tritium release in the supernatant was measured after 3 days with Cytoscint (ICN) as the scintillation fluid.

Each sample was tested in 4 replicates in each experiment. The well to well variation of the replicates was usually less than ± 20% of the mean value. The mean tritium release of the replicates was used to calculate the percent specific lysis, using the formula: (mean sample release - spontaneous release) / (maximum release - spontaneous release). To measure spontaneous release, labeled target cells were incubated in medium alone, and the supernatant was counted after 3 days. Spontaneous release of tritium from the target cells (in the absence of effector cells) averaged less than 10% in all experiments. Neither antibodies nor CSF-1 increased spontaneous lysis when incubated with the target cells alone. To measure maximum release, labeled target cells were lysed in a final concentration of 0.5% SDS.

The ability of the heteroconjugate 113F1 F(ab')₂-3G8 F(ab')₂ at 1 µg/ml to mediate lysis with AMC from two donors is shown in Figure 2. When AMC plus the heteroconjugate were tested against SK-Br-3 at an E:T ratio of 20:1, the average

antibody dependent lysis observed was 28%. As shown in Figure 2, when the effector cells were preincubated with 14 ng/ml of CSF-1, heteroconjugate mediated lysis was enhanced by approximately 110%.

Adherent cells from a total of 11 donors were tested with the F(ab')₂ fragment of bispecific antibody 2B1. To avoid the possible involvements of FcRI and FcRII on human AMC, 2B1 F(ab')₂ was used in the study instead of whole 2B1. (An experiment was also conducted to show that the F(ab')₂ fragments of the 2 parental antibodies of 2B1; 520C9 and 3G8, did not mediate any specific lysis whereas 2B1 F(ab')₂ caused almost complete lysis of the target cells.) The effects of CSF-1 at 10-100 ng/ml were studied to observe whether CSF-1 was effective in augmenting lysis mediated by 2B1 F(ab')₂. AMC preincubated with 14 to 100 ng/ml of CSF-1 gave higher specific lysis than AMC preincubation without CSF-1 (Figure 3). The total amount of target cell lysis obtained varied from donor to donor, although increases in specific lytic activity with CSF-1 treatment were reproducibly observed in all monocyte preparations. As higher levels of CSF-1 may be necessary to stimulate the AMC of minimally responsive or unresponsive donors, CSF-1 dose response studies were conducted using 100 ng/ml of 2B1 F(ab')₂ and the results are shown in Figure 4. Preincubating AMC with increasing concentrations of CSF-1, prior to introduction of 2B1 F(ab')₂, gave higher specific lysis starting at 10 ng/ml and did not yet plateau at 100 ng/ml in all donors.

In Vivo Test of CSF-1 for Anti-Tumor Efficacy

A. Meth A Sarcoma Model

Recombinantly produced CSF-1 (C 158) from CV-1 cell line (LAL assay: 2 ng LPS/ml, 8 ng LPS/mg CSF, 2 x 10⁷ U/mg) was injected i.p. at 50 µg/dose twice a day for five days into a 20 g mouse (3 mice per group) implanted subcutaneously with a Meth A sarcoma tumor 7 days earlier. For 6 days after the beginning of the CSF-1 treatment, the 3 untreated and 3 treated mice were evaluated for body weights and tumor volumes. There was no evidence of toxicity as measured by change in body weight. On day 7, one mouse from each group was sacrificed for comparative histopathological analysis (no gross signs). The four remaining mice were evaluated for the usual 14-day period in the Meth A model. The results are provided in Table 4 below:

Table 4

Day	Treatment		Percent
	CSF-1	Buffered Saline	$\frac{\Delta \text{TV Treated}}{\Delta \text{TV Control}}$
Mean Change in Tumor Volume (ΔTV)			
3	2.0	2.2	91
6	2.6	6.8	38
7	4.1	8.0	51
8	5.7	11.0	52
14	13.9	29.4	47

$\Delta \text{TV} =$ Ratio of the mean tumor volume at the day indicated to the mean tumor volume at day 0 within a single group of mice.

The results show that there was evidence for CSF-1-mediated efficacy, particularly at the day 6 tumor volume measurements. The differences between the CSF-1 and control groups were greatest during a period starting several days after the commencement of treatment and several days thereafter, after which the tumor returned to its usual rate of growth. These data suggest that multiple daily dosing (continuous infusions to improve efficacy at this dose level, for longer periods of time) or a higher dose level and altered schedule to include drug holiday may enhance efficacy.

Similar results were seen using LCSF C▼190, and LCSF/C▼221 from E. coli. The protocol was performed using a group of 5 mice; 50 $\mu\text{g}/\text{dose}$ of each product were used and the administration (twice daily for 5 days) was similar except for the E. coli C▼150 and C▼190 material wherein the schedule was increased to 10 days and administration was 7-8 hours apart. Table 5 provides results as a percentage of ΔTV Treated divided by ΔTV Control for each CSF-1-derived material.

Table 5

	Day	CV-1 (C▼158)	C▼150	C▼190	C▼221	N▼3 C▼221
5	3	21	0	0	36	14
	4	23	0	0	38	46
	5	46	5	--	44	48
10	6	59	7	57	50	40
	7	61	15	70	61	40
	8	64	7	81	40	39
	13/14	56	30	28	20	26

15

B. B16 Metastases Model

CSF-1 was tested in the B16 experimental metastasis model to assess its effect on the prevention of pulmonary metastases.

20 1×10^5 tumor cells, suspended in 0.2 ml of Ca^{+2} and Mg^{+2} -free HBSS, were inoculated into unanesthetized mice in the lateral tail vein. Twenty-one days after tumor cell inoculation, the mice were sacrificed and necropsied. During necropsy, the lungs and brain were removed, rinsed in water, and weighed. The lungs were then fixed in Bouin's solution, and the number of surface tumor nodules per pair of lungs was determined with the aid of a dissecting scope.

25

Recombinant human CSF-1 (N▼3C 221), was used for all experiments.

CSF-1 was freshly obtained prior to each experiment from frozen stocks and diluted immediately prior to injection in USP 0.9% saline. CSF-1 was delivered intravenously on a once a day (QD) x 10 day schedule. The dosing levels used are given in the following table. As a negative control consisting of a non-specific and non-therapeutic protein, either USP human serum albumin (HSA) or boiled CSF-1 was used. CSF-1 was boiled for 30 minutes to inactivate the CSF-1 activity.

30

The efficacy data shown in Table 6 demonstrates that CSF-1 given QD x 10, intravenously, starting 3 days before intravenous inoculation of 1×10^5 tumor cells produces a significant reduction in the median number of pulmonary metastases. In contrast, if the CSF-1 therapy was initiated one day post tumor cell inoculation, no significant decrease in the median number of pulmonary metastases was observed. No overt toxicity, as measured by lethality, was observed at this dose level (2.5-5.0 mg/kg).

35

Table 6

5	Group	Dose	Day of Initiation of Therapy	Median Number of Pulmonary Metastases (Range)
10	1. Saline	--	+1	55 (5, 29, 48, 52, 52 58, 58, 74, 80, 91)
15	2. M-CSF	2.5 mg/kg	+1	38 (11, 11, 13, 28, 32, 44, 50, 64, 67, 90)
15	3. M-CSF	5 mg/kg	+1	50 (22, 32, 48, 48, 48, 52, 57, 62, 65, 76)
20	4. M-CSF	2.5 mg/kg	-3	7 ^a (0, 0, 2, 5, 6, 8, 9, 11, 12, 19)

^a Difference is significant between this group and control at $p = 0.002$ (Mann-Whitney).

25 In a second experiment, CSF-1 was administered i.v. QD x 5. B16-W10 tumor cells were harvested by a brief one min trypsinization, centrifuged, and then prepared as a single cell suspension in Ca- and Mg-free HBSS. On day 0, 8×10^4 cells were injected per mouse, in a total volume of 0.2 ml in the lateral tail vein. CSF-1 (*E. coli* N ∇ 3C ∇ 221) therapy (0.25 to 5.0 mg/kg/day) was administered QD x 5, i.v.,

30 beginning on day -3. On day 14 the mice were sacrificed, the lungs removed, rinsed in water, and then fixed on Bouins fixative. Surface tumor nodules were counted with the aid of a dissecting scope. As shown below in Table 7, CSF-1 was able to significantly decrease the median number of pulmonary metastases when administered i.v. at either 1.0, 2.5 or 5.0 mg/kg/day, QD x 5.

Table 7

	Group	Dose (mg/kg/day)	Median	Number of Lung Metastases (individual values)	p-VALUE
5	HSA	5.0	199.5	(94,142,176,181,187, 212,221,223,236,250)	--
10	CSF-1	5.0	27.0	(2,4,11,18,25,29,31 33,39,98)	0.000
15	CSF-1	2.5	132.0	(12,33,43,67,105,159, 161,178,206,239)	0.034
	CSF-1	1.0	85.5	(19,61,62,64,64,107, 114,160,201,234)	0.013
20	CSF-1	0.5	173.5	(23,114,127,159,171, 176,191,194,200,236)	0.173

25 80,000 tumor cells i.v. on day 0
 Treatment: HSA or M-CSF QDx5 i.v. beginning day 3
 10 BDF-1 mice/group, sacrificed on day 14

30 The following experiment shows that CSF-1 may be administered by the
 subcutaneous (s.c.) or intraperitoneal (i.p.) route which are equally effective as CSF-1
 when administered i.v.

35 Tumor cells were prepared as taught above. On day 0, 7.5×10^4 cells were
 injected per mouse (5-10 female BDF-1 mice/group), in a total volume of 0.2 ml in the
 lateral vein. CSF-1 at 5 mg/kg/day, QD x 5 beginning on day -3 was administered by
 the three different routes. On day 18 the mice were sacrificed and the lungs were
 prepared as taught above. The results are shown below in Table 8.

Table 8

	Group ^a	Route	Number of Lung Metastases	
			Median	Individual values
5	1. HSA	i.v.	26.5	0,1,19,20,26,27,35,43,59,76
10	2. M-CSF	i.v.	1.0	0,0,1,10,30
	3. HSA	i.p.	22.0	16,17,22,33,34
	4. M-CSF	i.p.	1.0	0,1,1,3,4 ^b
15	5. M-CSF	s.c.	2.0	0,1,2,2,9 ^b
20	6. HSA	i.v.	85.5	2,8,23,30,50,61,64,68,73,84,87,91,91,93,98,112,147,150,150,150
	7. M-CSF	i.v.	6.0	0,1,2,5,5,7,7,13,15,18 ^b
25	8. M-CSF	i.v.	2.0	0,0,0,1,1,3,4,4,6,33 ^b
	9. M-CSF	s.c.	2.5	0,0,0,2,2,3,3,4,4,72 ^b
30	^a Groups 6-9 were run in a separate experiment from Groups 1-5 ^b p-value is less than 0.05 compared to the proper HSA control			

The following experiment compares the efficacy of CSF-1 administered by continuous infusion performed subcutaneously using Alzet osmotic pumps and subcutaneous bolus dosing. CSF-1 was administered either as a s.c. bolus or as a s.c. continuous infusion in 0.9% NaCl. Continuous infusions were performed using s.c. implanted Alzet pumps, models 1003D and 2001, which delivered CSF-1 for either 3 days or 14 days, respectively. The 1003D model pump has a mean pumping rate of 1 µl/hour and a mean fill volume of 87 µl. The 2001 model pump has a mean pumping rate of 0.417 µl/hour and a mean fill volume of 207 µl. For pump implantation, BDF-1 female (18-20 g) were anesthetized with Metofane and a small dorsal incision was made in the skin. Pumps were implanted under the skin with the flow moderator pointing away from the incision. The incision was closed with a wound clip. All therapy was initiated on day -3.

On day 0, to 7.5×10^4 B16-W10 tumor cells (prepared as previously described) were injected per mouse, in a total volume of 0.2 ml in the lateral tail vein using a 27-gauge needle. On day 18, the mice were sacrificed, the lungs removed, rinsed in water, and then fixed in Bouins fixative.

5 As shown in Table 9, CSF-1 administered by s.c. continuous infusion at doses as low as 0.25 mg/kg/day was highly effective at decreasing the median number of pulmonary metastases.

10 These studies suggest that CSF-1 when administered by s.c. continuous infusion is at least 10-fold more potent than the same dose given over the same period of time by once a day s.c. boluses.

Table 9

Group	Daily Dose mg/kg	Route*	Schedule	Lung Metastases		p-Value		
				Median	Individual value	vs HSA	vs s.c. bolus	
15								
20	1. HSA	1.0	s.c.	QDx14 d-3	58.5	21,27,54,58,59,66,71, >100, 100	--	--
	2. HSA	1.0	14d pump	d-3	56.0	2,40,40,42,44,68,69, 87,>100, 100	--	0.820
25	3. CSF-1	1.0	s.c.	QDx14 d-3	37.0	4,25,26,28,33,41,42, 75,>100,100	0.305	--
	4. CSF-1	1.0	14d pump	d-3	9.5	2,5,6,7,9,10,21,21,24,36	0.002	0.005
30	5. CSF-1	0.5	s.c.	QDx14 d-3	66.5	28,32,36,45,65,68,81, >100,100,100	0.849	--
	6. CSF-1	0.5	14d pump	d-3	19.0	2,11,15,16,19,20,25, 34,35	0.002	0.000
35	7. CSF-1	0.25	14d pump	d-3	14.0	0,5,8,10,11,17,20,25, 28,29	0.005	--
	8. CSF-1	5.0	i.v.	QDx5 d-3	12.0	0,0,2,10,11,13,14,15 52,91	0.015	--
40								

* Mice were implanted with Alzet osmotic pumps subcutaneously.

In Vitro Test of CSF-1 Alone and with IFN- γ for Anti-Tumor Efficacy

45 The present example tested a number of cytokines, besides CSF-1 and IFN- γ , for enhancement of tumor cell cytotoxicity.

Mononuclear cells (MNC) were separated from either heparinized venous blood or buffy coats of normal healthy volunteers by density gradient centrifugation on lymphocyte separation medium (LSM-Organon Teknika Corp., Durham, N.C.). MNC

were then washed twice with PBS and layered on 49.2% isotonic Percoll (Pharmacia) and centrifuged for 25 minutes at 1500 x g. The monocyte band at the interface ($\geq 80\%$ pure monocytes by morphological analysis of cyto-centrifuge preparations) was harvested and further purified by plastic adherence at 37°C. Adherence was done in 96 well plates at a density of 1.2×10^5 cells per well. After one hour, non-adherent cells were removed by vigorous washing, leaving approximately 1×10^5 cells per well.

Purified monocytes were cultured for 3 days in 0.1% FCS containing either CSF-1 (*E. coli* N ∇ 3C ∇ 221), IL-1, IL-3, IL-4, GM-CSF (all from Genzyme Corp.), IL-2 (Cetus Corp.), or medium alone (1° induction). After 3 days, monocytes were washed and then incubated for an additional 2 days with 2° inducers. 1° induction with or without CSF-1 was also carried out in flasks in several experiments. Monocytes were adhered directly in tissue culture flasks, non-adherent cells were removed, and 1° inducers were added. After 1° induction, monocytes were harvested by trypsinization and gentle scraping; viable cell counts were done by trypan blue exclusion, and 1×10^5 cells per well were plated in 96 well plates for the remaining 2 days of the protocol.

The WEHI 164 tumoricidal assay (Colotta *et al.*, *J. Immunol.* (1984) 132:936) was used to test the cytotoxicity of the cytokines. Briefly, WEHI 164 target cells in active log phase were either pre-treated for 3 hours with actinomycin D (act D) at 1 $\mu\text{g/ml}$, washed, and labeled for 1 hour with 200 μCi of ^{51}Cr , or treated simultaneously with act D and ^{51}Cr for 1 hour at 37°C in 5% CO_2 . After removing 100 μl of culture supernatant from the monocytes, labeled target cells were added in a 100 μl volume to the effector cells to achieve an effector to target ratio of 10:1, unless otherwise noted. The cells, in a volume of 200 μl , were allowed to incubate for 6 hours at 37°C in 5% CO_2 . The plates were then centrifuged for 5 minutes at 1200 rpm in a table-top swinging bucket centrifuge. 100 μl of supernatant was removed from each well and counted in a gamma counter.

P815 target cells were treated similarly, except the actinomycin D pre-treatment was omitted, and the target cells and effector cells were co-incubated for 18 hours.

Percent induced cytotoxicity was calculated using the formula:

30

$$\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

35

where: experimental cpm = effector cells + target cells + inducers
 spontaneous cpm = effector cells + target cells + media
 maximum cpm = target cells alone lysed with 1% SDS

The results are shown in Tables 10 and 11:

Table 10Percent Cytotoxicity Induced by CSF-1

5	Induction Protocol			
	1° 2°	Media CSF-1	CSF-1 CSF-1	CSF-1 Media
10				
	Experiment 1	19%	34%	35%
	Experiment 2	12%	49%	37%
	Experiment 3	4%	8%	5%
15	Experiment 4	7%	19%	9%

Although the level of activation induced by CSF-1 was variable, 16 out of 40 such donors showed between 10% and 49% enhanced tumoricidal activity upon stimulation by CSF-1 alone.

20 Table 11, CSF-1 was used as a 1° inducer prior to addition of a variety of 2° inducers.

Table 11

Percent of Cytotoxicity

	2° Stimulation	1° Stimulation	M-CSF at 1000 U/ml
5	Medium Control	0	2
	M-CSF 1000 U/ml	1	11
10	LPS 1 µg/ml	4	26
	IFN-γ 1 U/ml	0	7
	IFN-γ 100 U/ml	2	13
15	LPS 1 µg/ml + IFN-γ (1 U/ml)	11	29
	LPS 1 µg/ml + IFN-γ (100 U/ml)	7	49
20	LPS 1 µg/ml + PMA 2 ng/ml	22	53
25	LPS 10 µg/ml + PMA 2 ng/ml	24	45
	IL-2 50 U/ml	2	5
30	IL-2 500 U/ml	2	7

Other factors tested as 2° inducers and found to have no effect with or without CSF-1 included IL-1, IL-3, IL-4 and GM-CSF at up to 500 U/ml.

35

In Vitro Stimulation of Murine Antiviral Activity

Adherent murine thioglycolate-elicited macrophages were incubated with CSF-1 for 3 days and infected with VSV overnight (Lee, M.T., et al., *J. Immunol.* (1987) 138:3019-3022). Table 12 shows crystal violet staining as measured by absorbance at

40 550 nm of cells remaining adherent.

Table 12

	Treatment	Absorbance (Mean) (S.D.)
5	Medium/No virus	0.346±0.02
	Medium + virus	0.170±0.02
	CSF-1, 1000 U/ml + virus	0.264±0.02
10	CSF-1, 2000 U/ml + virus	0.356±0.04

CSF-1 treated cells, therefore, showed protection of the macrophage against VSV.

In Vivo Treatment of CMV Infection with CSF-1

15 Outbred CD-1 mice were treated with the CSF-1 (C 158) produced from the CV-1 cell line at doses of 400 µg/kg, i.p., once a day for 5 days, starting 2 days before infection with a sub-lethal dose of cytomegalovirus (CMV). Mice were sacrificed on the third day after infection and the extent of viral replication in target organs such as the spleen was evaluated by plaque assay. The results showed that mice treated with
20 CSF-1 have significantly lowered (57.8% reduction in) spleen viral titer compared to the saline-treated control mice, indicating that CMV infection is less severe in CSF-1-treated mice.

In a second experiment, 20 g Balb/C mice (5 per group) were infected with a sublethal dose of mCMV (2×10^4 pfu/mouse, i.p.) 4 hours after the last CSF-1 dosing.
25 CSF-1 was administered, i.p., once a day for 4 days at 4 dose levels (3.6, 0.9, 0.23 and 0.06 mg/kg/day). In this subacute infection model, mice were assessed for severity of infection by assaying viral titers (plaque forming units on mouse embryo cells) in blood and organs (spleen, liver and kidney) at 7 days after infection. CSF-1-pretreated mice showed 75-97% reduction in viral titers in spleens, kidneys and
30 livers as compared to saline treated control ($P < 0.01$). The CSF-1 effect is dose dependent; mean spleen viral titer reduction of 97.8, 95.3, 80.9 and 63.1% at CSF-1 dose levels of 3.6, 0.9, 0.23 and 0.06 mg/kg/day, respectively were observed as shown in Table 13:

Table 13
Dose Response of CSF-1 Effect on mCMV Organ Titer

5	Mean Viral Titer (% Decrease) ^a			
	CSF-1 Dose (mg/kg/day)	SPLEEN x10 ⁴ pfu/g	LIVER x10 ² pfu/g	KIDNEY x10 ² pfu/g
10	3.6	16.0±2.1(97.8) ^b	17.5±2.5(92.1) ^b	2.5±2.5(95.7) ^b
	0.9	32.9±6.4(95.3) ^b	35.0±10.0(84.3) ^b	17.5±2.5(69.6) ^b
	0.23	135.0±25.0(80.9) ^c	97.5±12.5(56.2) ^c	7.5±7.5(87.0) ^c
	0.06	260.0±25.0(63.1)	90.0±5.0(59.6) ^b	2.5±0(56.5)
15	HSA/saline	705.0±41.7	222.5±47.5	57.5±12.5

a = Data represent mean organ titer from five animals assayed individually. Value in parenthesis refers to percent decrease in organ titer as compared to HSA saline control.

b = p<0.01

c = p<0.05

20 Separately, CSF-1 produced in *E. coli* (N ∇ 3C ∇ 221) has been tested in a lethal
 25 mCMV infection model (this is in contrast to the above experiment using sublethal
 doses of CMV, in which organ titers were monitored). When 50 µg/ 0.05 ml CSF-1
 was administered i.p. to 13.5-14.5 g Balb/C mice at day -1 or day -1, 0, 1, 2 and 3
 (single dose given per mouse) before viral challenge (4 x 10⁵ pfu/mouse, 0.2 ml i.p.),
 after 7 days there was a significant increase in survival, shown in Table 14, as
 30 compared to saline-treated control.

Table 14

	<u>Group</u>	<u>Percent Survival</u>
35	Controls	3/10
	CSF-1 day-1	8/10
40	CSF-1 days -1,0,1,2,3	7/10

For prophylactic effect in the acute infection model, 13-14 g Balb/C mice were
 infected with a lethal dose of mCMV (5 x 10⁴ to 2 x 10⁵ pfu/mouse, i.p.) 4 hours after
 the last CSF-1 dosing (using the dose levels between 0.9 mg/kg/day up to 7.2

mg/kg/day) and survival monitored for 14 days. In this acute infection model, CSF-1 significantly enhanced survival in mice challenged with mCMV at 50,000 pfu/mouse as shown in Figure 5, although lower doses of CSF-1 (0.9 mg/kg and 3.6 mg/kg) appeared to be less effective in mice challenged with mCMV at 100,000 pfu/mouse.

5 These data demonstrate that CSF-1 can be used as an immunoprophylaxis for viral infection in clinical medicine. CSF-1 may be used alone or in combination with another lymphokine or antiviral agent in the treatment of viral infections in general, and in particular, may be beneficial in immunosuppressive viral infections such as acquired immune deficiency syndrome (AIDS).

10 The preferred dosage range administered i.p. is about 0.05-8.0 mg/kg CSF-1 per mouse per day.

In Vivo Prophylactic Treatment of Bacterial Infection with CSF-1

Outbred CD-1 mice were individually administered CSF-1, produced from the CV-1 cell line (short clone C▼158), one dose (10 µg/dose), administered i.p. 1 day
15 before challenge with a lethal dose ($LD_{100} = 6 \times 10^7$ cfu) of a clinical isolate of *E. coli* (SM18), a bacterium responsible for causing Gram-negative sepsis upon introduction into a host. The mice were then monitored for survival for 7 days post-infection. The data show that pretreatment with CSF-1 enhanced the survival of mice challenged with lethal doses of *E. coli*.

20 This experiment was also conducted using the LCSF N▼3C▼221 bacterially produced CSF-1. Each lot of CSF-1 was tested in 4-fold dilutions for a total dose range of 1×10^7 to 1.7×10^8 units/kg body weight. Minimum protective dose was defined as single daily dose (administered once a day for 5 days, i.p.) before i.p. infection with *E. coli* (6×10^7 cfu/mouse) which produced statistically significant (p
25 value less than 0.05 Fisher's Exact test) enhancement of the survival of treated mice as compared to saline or boiled CSF-1 control mice. The results are shown in Table 15.

Table 15

	<u>Group</u>	<u>Dose</u>	<u>Percent Survival^a</u>
	Saline	--	0
30	CSF-1	2.97 mg/kg	100
	CSF-1	0.74 mg/kg	90
	CSF-1	0.18 mg/kg	10
	Boiled ^b CSF-1	2.97 mg/kg	0
35	Boiled CSF-1	0.74 mg/kg	20
	Boiled CSF-1	0.18 mg/kg	0

^a Data represent percent of animals surviving at day 7 after infection.

^b Heat inactivated controls.

Experiments were conducted to study the effect of dose scheduling on the induction of CSF-1 effects on host resistance. Groups of mice (10) were administered CSF-1 at 0.9 mg/kg/dose per day for either 1, 2, 3, 4 or 5 days. Mice were then challenged with *E. coli* (6×10^7 cfu/mouse) i.p. 4 hrs after the last CSF-1 injection.

- 5 To induce a protective effect the data shown in Table 16 indicate that multiple doses of CSF-1 starting between 52 and 100 hours before bacterial infection are effective.

Table 16

10	CSF-1 ^a Dose Schedule	Time Before ^b Infection	Percent Survival ^c Day 7	p ^d Value
	QD x 1	4	10	--
15	QD x 2	28	10	
	QD x 3	52	60	<0.05
	QD x 4	76	90	<0.01
	QD x 5	100	100	<0.01
20	Saline	76	0	--

^a = Groups of 10 mice were treated with CSF-1, i.p. (at 0.9 mg/kg/dose/day, for one, two, three, four or five days (i.e. QD X 1 to QD X 5) before *E. coli* infection at four hours after the last CSF-1 dose.

25 ^b = Duration in hours between the first dose of CSF-1 and the time of infection with *E. coli*.

^c = Data represent percent of animal surviving at 7 days after infection.

30 ^d = By Fisher's Exact test, as compared with the saline control group.

Single bolus injection (0.2 to 9.0 mg/kg) at either 4, 18, 28, 52 or 76 hrs before infection was not effective at inducing enhanced host resistance.

35 The data show that pretreatment with CSF-1 significantly enhances survival of mice challenged with lethal doses of *E. coli*. The effect is dependent, however, on the dose of CSF-1, the timing, and the schedule of administration. At the higher doses of approximately 0.7 to 3.0 mg/kg/day, nearly complete protection was seen. At the lower dose of 0.2 mg/kg, there was also protection but the effect was smaller.

40

Leukopenic Infection Model

CSF-1 induced protection in *E. coli* infection in mice pretreated with 50 mg/kg cyclophosphamide (CY). The LD₅₀ of CY for mice is at about 400 mg/kg, the lower CY dose used represents 1/8 of the LD₅₀. This dose, when injected i.p. 3 days earlier,

induced a decrease in total white blood cells and neutrophils, and rendered mice more susceptible to *E. coli* infection (e.g., infection with 3×10^7 cfu/mouse killed 100% of CY treated mice but only 20% of mice not given this dose of CY). When CSF-1 was given to CY treated mice (CSF-1 at 0.89 mg/kg, once/day for 4 days, i.p.), there was 100% survival as compared to 30% in mice given saline instead.

In Vivo Effect of CSF-1 on Candida Albicans

CSF-1 was administered to outbred CD1 mice (27-28g, females) as daily i.p. doses for 3 to 4 days before challenge with a lethal dose of *C. albicans* (1.5×10^8 yeast cells/mouse, i.p.). This challenge dose resulted in a median survival time (MST) of 3.0 days in non-treated and saline treated mice. CSF-1 treated mice showed MST of 15 and 13 days at doses of 1.9 and 0.1 mg/kg, respectively. This 4-fold enhancement in survival time is significant at $p=0.01$ (Log Range Tests). To minimize any possible interference from endotoxin, highly purified CSF-1 was used which was virtually free of endotoxin (<0.05 ng/mg protein). It was also shown that the prophylactic effect was abrogated by heat-inactivation of CSF-1 test material. This protection was associated with increased numbers of peripheral blood circulating monocytes and neutrophils and a 2- to 3-fold increase in peritoneal macrophages. Therefore, CSF-1 enhanced host resistance against *C. albicans* infection and that this effect is probably mediated by activation of macrophages and neutrophils.

CSF-1 was also tested in an additional model in which *C. albicans* was delivered systemically (i.v.). CSF-1 at a dose of 1.9 mg/kg/day QD x 4 was administered either i.p. or i.v. 2×10^5 cfu/mouse were injected i.v. 4 hours after the last dose of CSF-1. Either of these administrations resulted in a significant enhancement of survival when compared to saline injected control mice.

Survival Time of Rats With Fungal Infections After CSF-1 Treatment

Using CSF-1 in conjunction with the antifungal agent fluconazole, we demonstrated that dailing bolus subcutaneous (SQ) injection of CSF-1 given therapeutically improved the median survival time of infected animals in a rat Candidiasis model from 5 days (in animals receiving fluconazole alone) to greater than 30 days (in animals receiving a combination of CSF-1 and fluconazole).

Male Fisher-344 rats weighing 200-250 grams were obtained from Charles River Laboratories. Rats were housed at 5 per cage and received water and laboratory chow *ad libitum*. Serological testing for adventitious viruses and mycoplasma is routinely negative in these animals.

Candida albicans strain ATCC No. 18804 from the American Type Culture Collection (Bethesda, MD) was grown overnight at 37°C in trypticase soy broth and then centrifuged. The blastospores were resuspended and aliquoted in 95% fetal bovine serum and 5% dimethyl sulfoxide and frozen at -80°C. On the day of each experiment two aliquots were pooled, and then diluted in phosphate buffered saline (PBS) to 4 x 10⁶ blastospores per mL for injection. Each experimental inoculum was plated on Yeast Extract Peptone agar to confirm the viable organism count.

Fisher-344 rats were infected with 2 x 10⁶ *Candida albicans* blastospores (0.5 mls) by i.v. injection into a lateral tail vein. In the CSF-1 efficacy studies, a single 0.3 mg/Kg dose of fluconazole (Diflucan, Pfizer, New York, NY) was administered subcutaneous (SQ) 2 hours later. Immediately following the fluconazole treatment, a 0.5 mL SQ dose of CSF-1 was given at a different site. CSF-1 treatment was continued for an additional 9 days for a total of 10 doses. Animals were assessed daily for mortality for 30 days.

The dose of *Candida albicans* needed to produce 100% mortality in untreated F-344 rats was determined to be 2 x 10⁶ blastospores (Table 17). This dose resulted in mortalities in 3 to 6 days, with a median survival time of 4 days.

Table 18 presents the results of four experiments in which fluconazole was combined with the therapeutic SQ administration of CSF-1 after *Candida albicans* infection. Doses of CSF-1 at 0.3 and 1.0 mg/Kg/day appear to be the most efficacious, resulting in a median survival time of >30 days, at least 6 times the median survival time of control animals (5 days).

Table 17

Mortality Table of Fisher 344 Rats Infected with *Canadida Albicans*

	No. of <i>C. albicans</i> Blastospores Injected (x 10 ⁻⁵)	No. of Animals Tested	Percent Overall Mortality	Median Survival Time (Days)	Mean Survival Time of Lethally Infected Animals (Days)
25	1.25	5	0	>30	
	2.5	5	20	>30	5.0
	5	16	38	>30	5.0
30	10	16	88	4	4.4
	20	50	100	4	4.4
	40	5	100	4	4.4
	100	8	100	2	2.8
	330	8	100	1	1.5
35	1000	4	100	1	1.0

Table 18

Therapeutic Use of CSF-1 with Fluconazole in *Candida Albicans* Infected Rats

CSF-1 Dose with 0.3 mg/kg Fluconazole*	Experiment Number Survivors/Total				Survival Totals from 4 Experiments	Median Survival Time (Days)	% Survival >30 days
	#1	#2	#3	#4			
CSF-1							
0.0 mg/kg	1/5	4/10	1/8	4/10	10/33	5	30%
0.03 mg/kg	ND	ND	1/5	ND	1/5	5	20%
0.1 mg/kg	3/5	ND	2/5	3/5	8/15	>30	53%
0.3 mg/kg	4/5	5/5	3/5	3/5	15/20	>30	75%
1.0 mg/kg	4/5	5/5	4/5	4/5	17/20	>30	85%
3.0 mg/kg	ND	5/5	1/5	3/5	9/15	>30	60%
10.0 mg/kg	ND	ND	ND	1/5	1/5	6	20%
Control-PBS (No fluconazole)	0/10	0/10	0/5	0/6	0/31	4	0%

* CSF-1 therapy of a SQ injection daily for 10 days began on Day 0, 2 hours post-infection. All test animals receive a single dose of fluconazole, 0.3 mg/kg, 2 hours post-infection.

ND = Not done.

Resolution of Fungal Infections Using CSF-1

Eighteen patients with invasive fungal infection which was refractory to standard therapy were treated with CSF-1. Those patients who were referred to a bone marrow transplant (BMT) center were eligible for study, but patients with a malignancy in relapse were ineligible. No exclusions were made for diagnosis, age, graft versus host disease (GVHD) status or patient's overall condition. The clinical characteristics of the 18 patients are shown in Table 19.

The diagnosis of invasive fungal infection required demonstration of organisms in biopsy specimens, cultures of closed body fluids (i.e., cerebral spinal fluid) or radiologic evidence for invasive disease in the presence of positive blood cultures. Demonstration of fungi solely in bronchoscopic lavage fluid, in gastrointestinal biopsy specimens, or in single blood cultures were considered insufficient proof of invasive disease. The infecting fungal organism, the organs involved, and diagnostic modalities used to follow the infections are shown in Table 19.

All patients were examined and had routine blood cultures taken daily, as well as having blood chemistries and complete blood cell counts performed daily. Generally, bone marrow aspirates and biopsies were performed prior to therapy to rule out malignancy. The fungal infections were re-evaluated using the initial diagnostic procedures on a periodic basis and at autopsy.

The study was designed as a Phase I dose escalation trial in which patients were enrolled consecutively. All patients were maintained on the best available anti-fungal

therapy at the maximally tolerated dose. CSF-1 (specific activity approximately $3\text{--}10 \times 10^7$ U/mg) was given at a dose between 0.05 to 2.0 mg/M². CSF-1 was administered in 100 ml of normal saline with 0.25% albumin by central venous catheter as a single two-hour intravenous infusion daily for seven days. If after seven days there was no evidence for a clinical response, the dose was doubled and administered for an additional seven days. Three dose escalations were allowed in a single patient. If antifungal efficacy was noted, CSF-1 was continued until resolution of the fungal infection could be documented. Patients treated with 2 mg/M² were maintained at that dose.

CSF-1 was well tolerated at all dose levels. There were no specific symptoms or changes in blood chemistries that could be ascribed to it. There were no significant adverse effects on the severity of preexisting GVHD during the CSF-1 infusion course.

Eleven patients had invasive candidiasis, four had aspergillosis, two were diagnosed with yeast sp., and one was diagnosed with Rhodotorula (Table 19). Three patients received CSF-1 prior to BMT for progressive fungal disease that had not responded to amphotericin B. One of these patients had undergone two sinus cavity resections for aspergillosis. However, CT scan demonstrated a residual sinus mass, and aspiration cultures continued to demonstrate aspergillus. When cavitating multi-lobar pulmonary nodules developed and the patient became progressively azotemic on 1 mg/kg of amphotericin, CSF-1 was started and the amphotericin dose was reduced to 0.5 mg/kg. After 35-days of therapy, the CT scan showed clearing of the sinus cavities and cultures for aspergillus became negative. All but the two largest cavitary pulmonary nodules resolved. Both pulmonary nodules were subsequently resected and shown to be free of fungal elements. The patient remains off antifungal therapy awaiting an unrelated donor BMT. The second patient treated prior to BMT received two grams of amphotericin for Candida albicans (involving the liver and spleen) without a clinical or radiologic response. After 35 doses of CSF-1, marked radiologic evidence of improvement was noted, and the liver was rebiopsied with no evidence of residual fungal infection. The third patient had progressive hepatic candidiasis. She had clinical and radiologic resolution of the fungal infection after receiving 28 doses of CSF-1.

Thirteen of the eighteen patients showed resolution or clinical improvement in their fungal infections (Table 19). Several patients had complete resolution of their fungal infections and were discharged from the hospital. Two patients were well enough to receive a bone marrow transplant after their infections cleared. Of the remaining five patients, one patient was not evaluable, another patient may not have had an invasive fungal infection and expired due to pancreatitis, and the remaining three patients showed no response, but received CSF-1 treatment for 8 days or less.

Table 19

Patient Age, Sex	Primary Disease	Transplant Type/Date	GVHD	Fungus	Site	Days M-CSF	Dose $\mu\text{g}/\text{M}^2$	Response/ Basis	Outcome
#1 7, ♂	ALL	Allogeneic	+/+	Candida sp	Liver	11	100	+ (autopsy)	Death: ARDS
#2 47, ♀	Hodgkins	Autologous	-	Candida sp	Lung Blood	7 28	100 200	+ (CT and clinical)	Survival
#3 24, ♂	Aplastic Anemia	Allogeneic	-	Candida sp	Lung	3	100	-	Death: Candida sepsis
#4 44, ♂	ALL	Allogeneic	+	Candida sp	Blood Lung	9	100	+ (autopsy)	Death: renal failure and VOD
#5 23, ♂	ALL	Not Planned	-	Aspergillus sp	Lung Sinus Cavity	7 7 23	200 500 1000	+ (CT and clinical)	Survival
#6 40, ♂	Lymphoma in relapse	Allogeneic	++	Candida sp	Liver/Spleen	7 7 7 5	50 200 500 1000	+ (CT and clinical)	Survival
#7 20, ♂	AML	Allogeneic	++/+++	Candida sp	Lung Blood	7 7 7	200 500 1000	+ (clinical)	Death: acute GVHD
#8 22, ♂	AML	Allogeneic	-	Candida parapsilosis	Blood Lung	10	500	+ (autopsy)	Death

Table 19 Cont.

Patient Age, Sex	Primary Disease	Transplant Type/Date	GVHD	Fungus	Site	Days M-CSF	Dose $\mu\text{g}/\text{m}^2$	Response/ Basis	Outcome
# 9 42, ♀	ALL	Autologous	N.A.	Candida tropicalis	CSF	7 4	500 1000	-	Death: cardiovascular collapse
# 10 27, ♂	AML	Allogeneic	++	Rhodotorula	blood	10	1000	-	Death: pancreatitis secondary to GVHD
# 11 39, ♂	NHL	Autologous	N.A.	Candida sp	+skin Bx	14	1000	+ (CT)	Survival
# 12 21, ♂	AML	Pre-transplant	N.A.	Candida sp	liver	7 1	1000 2000	+ (CT/clinical)	Survival
# 13 12, ♂	Aplastic anemia	Allogeneic	+	Candida sp	liver/spleen/kidney	7 26	1500 2000	+ (clinical)	Survival
# 14 43, ♀	AML	Autologous	N.A.	Yeast sp	Blood Lung	8	1500	-	Death: renal failure secondary to fungal sepsis
# 15 25, ♀	AA	Pre-transplant	N.A.	Yeast sp	liver	30	2000	+ (CT/clinical)	Survival
# 16 8, ♂	AM	Allogeneic	+++	Aspergillus sp	Sputum tracheal/lung	28	2000	+ (CT/clinical)	Survival
# 17 52, ♂	ANL MDS	Allogeneic	++/+++	Aspergillus sp	RUL-lung	14	2000	Not evaluable	Death: myocardial infarction
# 18 7, ♂	ANL	Allogeneic	+++	Aspergillus sp	Skin Disseminated	27	2000	+ (CT/clinical)	Death: CMV pneumonia and acute GVHD

In Vivo Stimulation of White Blood Cell Count

Outbred CD-1 mice were administered purified recombinant human CSF-1, at 2 mg/kg per dose, three times a day for five consecutive days. Total white blood cell count increased to 12,000-13,000/ μ l in CSF-1-treated mice from 8,700/ μ l in
5 saline-treated control mice. In addition, neutrophil count increased to 6,821/ μ l in CSF-1-treated mice as compared to 1,078/ μ l in saline-treated control mice.

This effect is dependent on the dose of CSF-1 and the schedule of administration. The increase in peripheral blood neutrophils was detectable 2-4 hours after a single dose of CSF-1 was administered i.p. These results indicate that CSF-1
10 administration may be useful in clinical or veterinary medicine as a stimulus of granulocyte production and an enhancer of white blood count.

CSF-1 in Wound Healing

CSF-1 is assayed for wound healing using animal models and protocols such
15 as the Goretex miniature wound healing model of Goodson and Hunt (J Surg. Res., 1982, 33:394) in which implanted Goretex tubes fill up with invading macrophages, fibroblasts and other connective tissue cells, and collagen and fibrin deposition. Healing is assessed by examining tube contents microscopically. A second model is the excisional wound healing model of Eisenger, et al., (1988, PNAS (USA), 85:1937)
20 in which wounds are observed visually and punch biopsies are taken to monitor healing, density of cells, and number of epidermal cell layers arising from hair follicles. A third model is a serosal model such as the heat-injured testicular serosa of Fotev, et al., (1987, J. Pathol., 151:209), in which healing is assessed in fixed sections by degree of mesothelial resurfacing of the injured site. The teachings of each of these
25 models are incorporated herein by reference.

Generally, CSF-1 is applied to the site of the wound by soaking a nonadhesive surgical dressing in 10^4 to 10^8 U/ml of CSF-1 in saline as described in the excisional wound healing model reference using epidermal cell derived factor (EGF) for topical wounds. Alternatively, similar amounts of CSF-1 are introduced into Goretex tubes at
30 the time of implantation as described in Goodson and Hunt, supra, or CSF-1 may also be incorporated into a slow-release matrix and applied at the site of the wound (in Goretex tubes, in or under dressings, in a slow release gelatin or collagen-based matrix or by injection in the peritoneal cavity) by systemic treatment 1-3 times a day (i.v., i.p., or s.c.) at a dose of 10 to 10,000 μ g/kg/day.

35 In a full thickness dermal excisional model, experimental groups of five female BDF-1 mice, weighing 18 to 20 grams, were anesthetized by methoxyflurane inhalation (Metofane, Pitman-Moore, Inc., Washington Crossing, NJ). Wounds were made

using a clean surgical technique. Hair on the dorsum and sides was clipped, and the skin swabbed with 70% ethanol and dried. A strip of transparent tape was applied over the back, approximately midway between the sacrum and scapulae. The skin was elevated parallel to the length of the mouse, and full thickness excisional wounds were created using a 6 mm diameter punch. The tape was removed, exposing an 8 to 10 mm wide strip of intact skin between the left and right circular bilateral wounds. Triple antibiotic ointment (polymyxin B- bacitracin-neomycin) was applied to the fresh wound using a cotton tipped swab stick.

Wounds were measured with hand held calipers in their anterior-posterior and transverse dimensions on days 0, 1, 2, 3, 4, 5, 7, 9 and 10 (with day 0 representing the day of wounding). Although the wounds were circular initially, they tended to heal in an elliptical shape. For this reason, approximate wound area was calculated using the formula for the area of an ellipse $A = \pi(B \times C)/4$, where A = area (mm²), B = wound diameter (mm) of the anterior-posterior axis, and C = wound diameter (mm) of the transverse axis. Percent wound closure was calculated for each wound by dividing the area at any given time point by initial wound area on day 0.

E. coli produced long clone CSF-1 N ∇ 3C ∇ 221 (specific activity $>6.0 \times 10^7$ units/mg) was diluted in 0.9% sodium chloride, USP, and delivered intravenously via the lateral tail vein in a final injection volume of 100 μ l. Doses of CSF-1 ranging from 0.5 mg/kg/day (10 μ g/day) to 10.0 mg/kg/day (200 μ g/day) were administered daily for a total of 7 days, with the first dose occurring approximately 4 hours after wounding. Human serum albumin (HSA) diluted in 0.9% NaCl was chosen as a non-specific protein control, and was administered to control animals at a dose of 5.0 mg/kg/day.

Statistical analysis was done by individual Student t test comparisons between treatment groups on each day. For all comparisons, statistical significance was noted when $p < .05$.

Little or no hemorrhage occurred in fresh wounds. A thin fibrinous covering was evident over wounds within 12-24 hours, progressing to a scab within 1-2 days. The scabs contracted as they dried, gradually becoming less adherent to the underlying granulation tissue, but did not distort the wound area measurements. Mean wound area was calculated from measurements of the right and left wounds of 5 mice for each group and day. Initial wound area did not differ among any of the treatment groups.

As shown in Figure 6, wound closure was more rapid in CSF-1 treated mice than in HSA treated controls, where closure was defined as the percent reduction in initial wound area on a given day. The values provided in Figure 6 represent the means of 5 mice at each time point. CSF-1 treated group differed from the controls at all time

points ($p < .05$). The "square" represents the control; the "+" represents CSF-1 (10.0 mg/kg/day); the "triangle" represents CSF-1 (5.0 mg/kg/day); and the "x" represents CSF-1 (0.5 mg/kg/day). The greatest enhancement in wound closure was observed in mice receiving 10.0 mg/kg/day. Intermediate (5.0 mg/kg/day) and low (0.5 mg/kg/day) doses of CSF-1 also significantly increased rates of wound closure, while at these two doses the response was nearly equivalent.

The enhanced rate of wound closure seen in CSF-1 treated wounds appeared to result from a more rapid initial phase which occurred within the first few days. During this period, closure was enhanced approximately 40% by CSF-1 as shown in Table 18. Thereafter, the rate of closure in all groups was nearly identical and began to decline steadily until the wounds were completely healed. Wounds of CSF-1 treated mice reached 50% closure 1 to 2 days sooner than HSA treated controls (Figure 6), yet the period of time required for wounds to reach 100% closure was approximately 10 days for all groups.

Table 20

Days Post-Wound	Control					*CSF-1		
	Wound Area (mm ²)	Standard Deviation (mm ²)	Wound Closure (%)	Wound Area (mm ²)	Standard Deviation (mm ²)	Wound Closure (%)	Enhanced* Closure (%)	P value Relative to Control
0	35.2	7.5	0.0	34.4	8.1	0.0	--	0.828
1	25.2	6.9	28.4	18.4	3.6	46.6	64.0	0.017
2	19.4	4.8	44.7	11.6	2.3	66.1	47.9	0.000
3	17.4	5.2	50.5	9.0	2.4	73.9	46.4	0.000
4	11.2	3.7	68.1	7.8	2.6	77.2	13.4	0.037
5	9.9	3.1	71.8	5.1	1.9	85.2	18.7	0.001
7	5.0	3.1	85.7	2.6	1.0	92.4	7.9	0.045
10	0.0	0.0	100.0	0.0	0.0	100.0	0.0	---

* CSF-1 was administered to mice intravenously at a dose of 5.0 mg/kg/day for a total of 7 days, beginning 4 hours after wounding.

* Enhanced closure calculated as:
$$\frac{\% \text{ closure (treated)} - \% \text{ closure (control)}}{\% \text{ closure (control)}}$$

Gross observations made at time points from day 3 to day 10 suggested that changes in the vascular content of the skin surrounding the wound space occurred during the course of healing in control and CSF-1 treated wounds. As early as 3 days post-wounding, increased vasculature (larger and more tortuous vessels) was evident by the naked eye in regions of skin surrounding the wound sites in control mice. By

day , the vascular content of skin surrounding control wounds had decreased while the skin surrounding the wounds in CSF-1 treated mice showed significantly greater vascularization than did the control mice, both in areas around the wound and in vessels not adjacent to the site of wounding. This response appeared to include both a greater
5 number of vessels and more extensive branching.

CSF-1 may also be used in combination with other growth factors to promote wound healing such as epidermal growth factor (EGF), fibroblast growth factor (basic and acidic FGF), platelet derived growth factor (PDGF) or transforming growth factors (TGF alpha and beta), IL-1, IL-2, platelet derived wound healing factor (PDWHF) and
10 other substances such as somatomedin C and vitamin C.

Formulation and Dosage of CSF-1

The recombinantly produced human CSF-1 may be formulated for administration using standard pharmaceutical procedures. Depending on ultimate indication, CSF-1 will be prepared in an injectable or a topical form, and may be used
15 either as the sole active ingredient, or in combination with other proteins or other compounds having complementary or similar activity. Such other compounds may include alternate antitumor, e.g., chemotherapeutic agents such as adriamycin, or lymphokines, such as IL-1, -2, -3, -4, and -6, alpha-, beta-, and gamma-interferons, CSF-GM and CSF-G, and tumor necrosis factor. The effect of the CSF-1 active
20 ingredient may be augmented or improved by the presence of such additional components. As described above, the CSF-1 may interact in beneficial ways with appropriate blood cells, and the compositions of the invention therefore include incubation mixtures of such cells with CSF-1, optionally in the presence of additional lymphokines or cytokines. Either the supernatant fractions of such incubation
25 mixtures, or the entire mixture containing the cells as well, may be used. Staggered timing may be preferred for some combinations, such as CSF-1 followed one to two days later by gamma interferon.

The CSF-1 described herein is generally administered therapeutically in amounts of between 0.01-10 mg/kg per day, whether single bolus administration or
30 fractionated over 24 hr, for all indications, e.g., treatment of infectious disease, wound healing, restoration of myelopoiesis and immunity, and cancer.

The scope of the invention is not to be construed as limited by the illustrative embodiments set forth herein, but is to be determined in accordance with the appended claims.

WE CLAIM:

1. A method for therapeutic treatment of fungal infection in humans which comprises administering to said human an effective amount of colony stimulating factor-1 (CSF-1) at a daily dose between 0.5 and 50 mg/M².
2. A method in accordance with claim 2, wherein the infection is caused by at least one fungus selected from the group consisting essentially of Candida, Aspergillus, Cryptococcus, Histoplasma, Coccidioides, Paracoccidioides, Mucor, Rhodotorula, Sporothrix, and Blastomyces.
3. The method of claim 2, wherein the fungus is Candida species.
4. The method of claim 1, wherein the recombinant CSF-1 is administered with one or more antifungal agents.
5. A method in accordance with claim 4, wherein the one or more antifungal agents are selected from the group consisting essentially of Amphotericin B, Fluconazole, 5 fluoro-cytosine, Ketoconazole, Miconazole, and Intraconazole.
6. A method in accordance with claim 1, wherein the CSF-1 is parenterally administered until the fungal infection is cleared.
7. A method in accordance with claim 6, wherein the CSF-1 is administered subcutaneously by bolus injection, by i.v. bolus, by constant infusion or by continuous infusion.
8. A method in accordance with claim 1, wherein the daily CSF-1 dose that is effective to treat fungal infections is between 0.5 to 10 mg/M².
9. A method in accordance with claim 8, wherein the daily CSF-1 dose that is effective to treat fungal infections is between 0.5 and 5 mg/M².
10. A method in accordance with claim 6, wherein the CSF-1 is administered for at least 14 days.

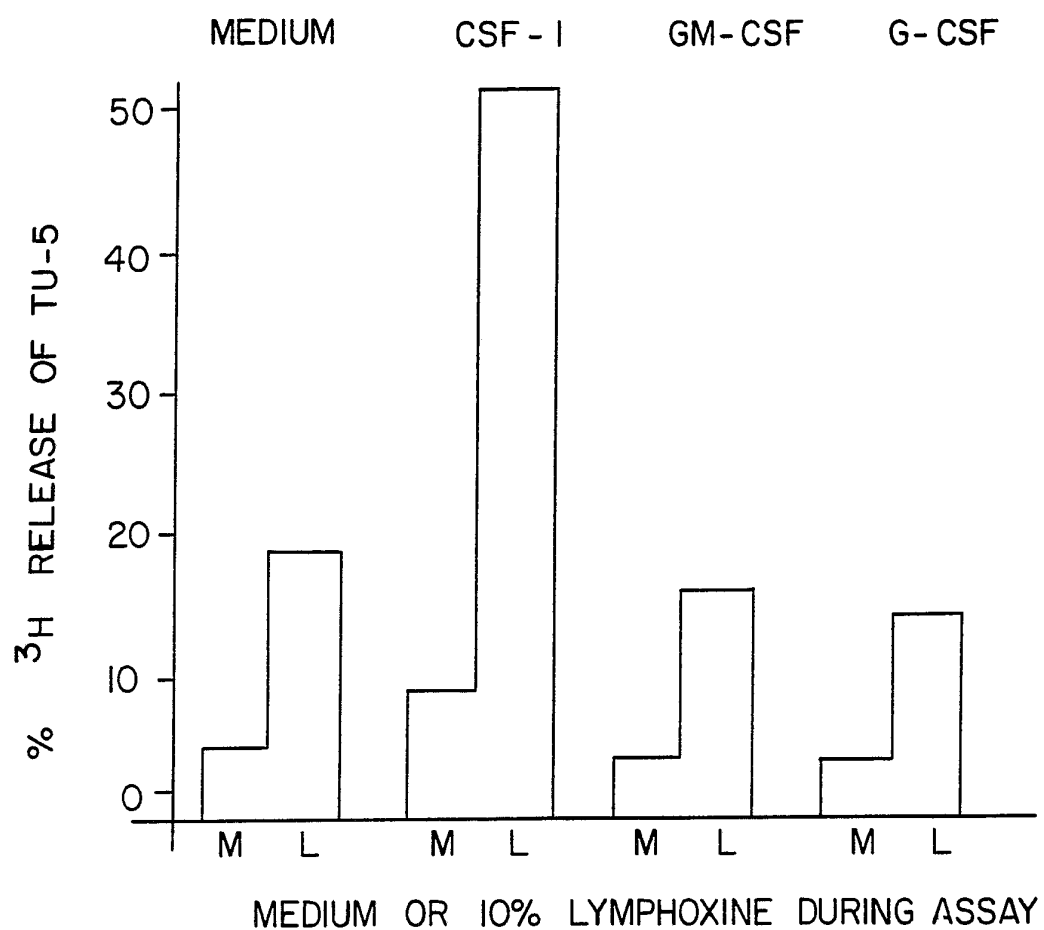
11. A method in accordance with claim 10, wherein the CSF-1 is administered for at least 21 days.

5 12. A method in accordance with claim 1, wherein the CSF-1 is administered to immunosuppressed individuals.

10 13. A method in accordance with claim 12, wherein the CSF-1 is administered to individuals who are immunosuppressed due to AIDS or other infections, due to the chemical agents that are given with a bone marrow transplant or in cancer chemotherapy or due to burns or other major trauma.

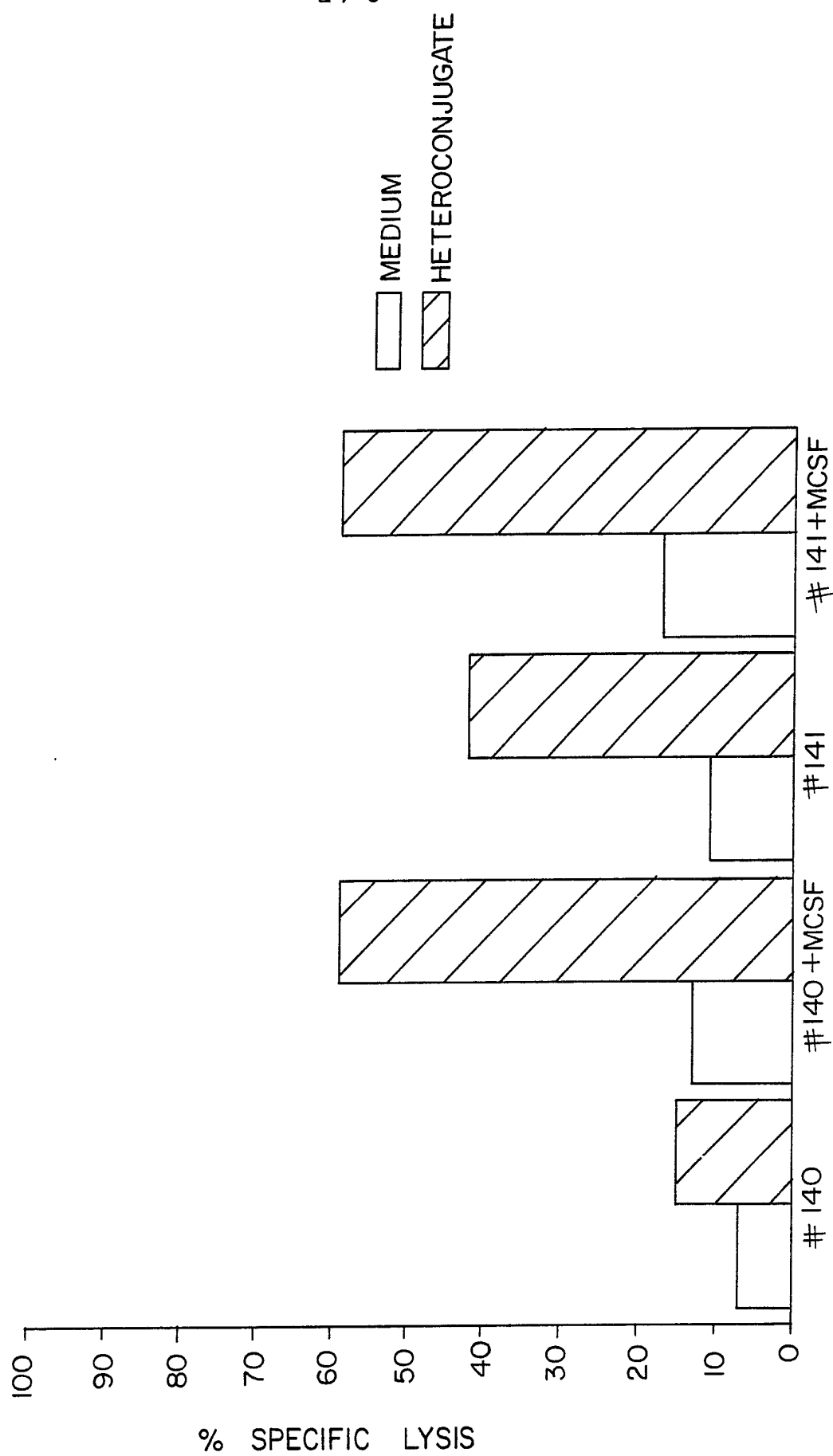
14. A method for treating mammals comprising administering CSF-1 to treat viral or bacterial infections, to promote wound healing, and to treat tumor burden.

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FIG. 1

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FIG. 2



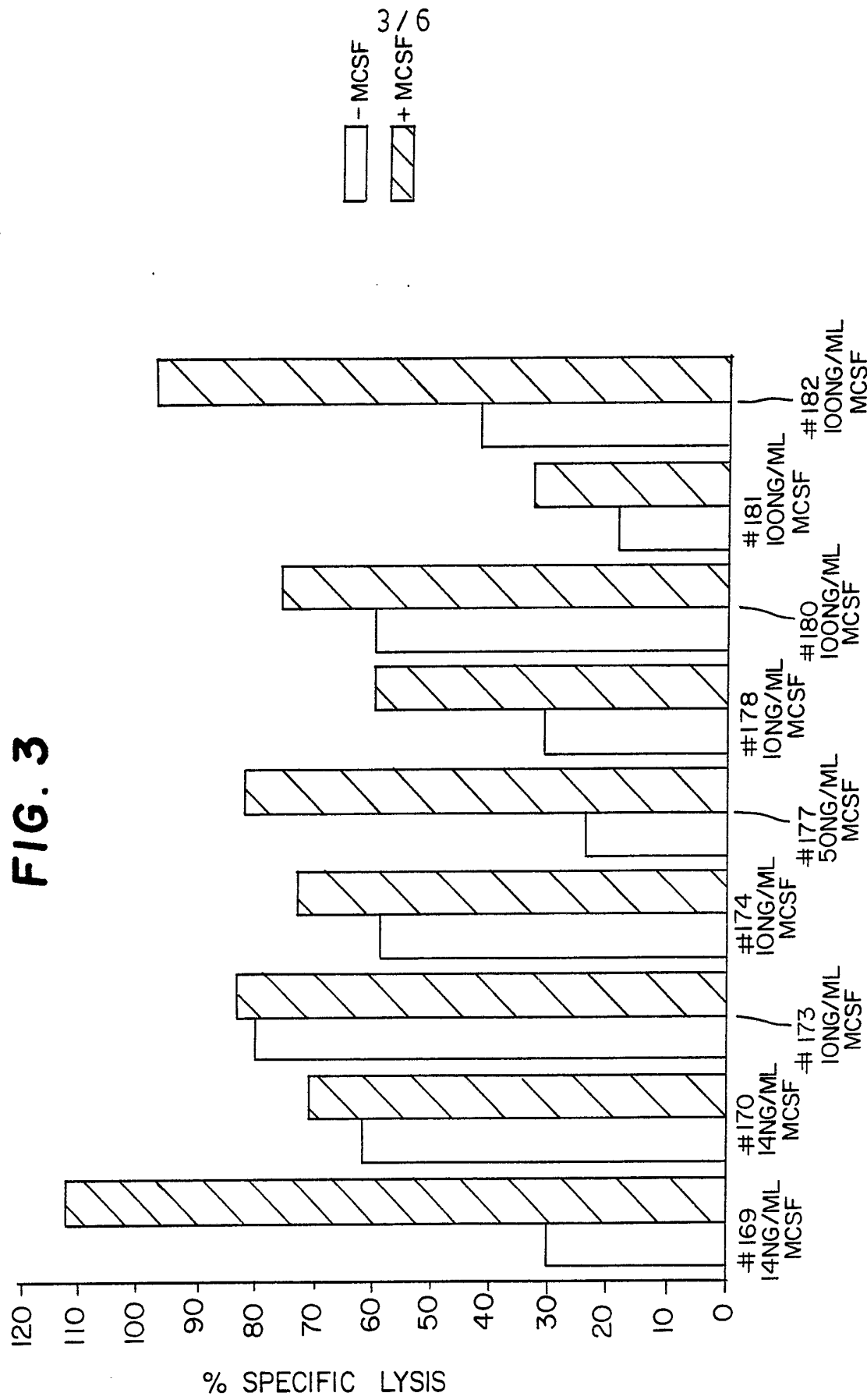
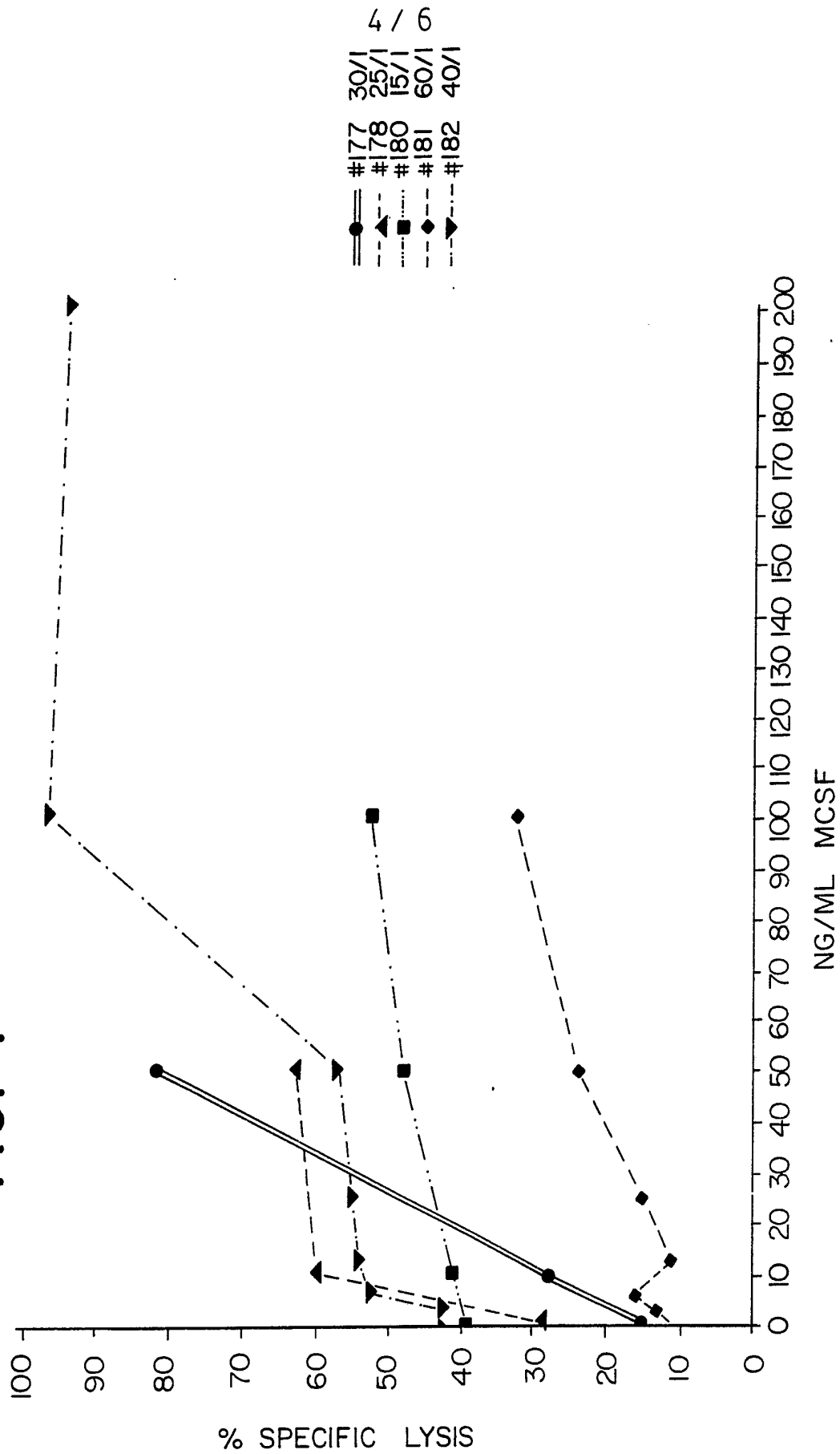


FIG. 4



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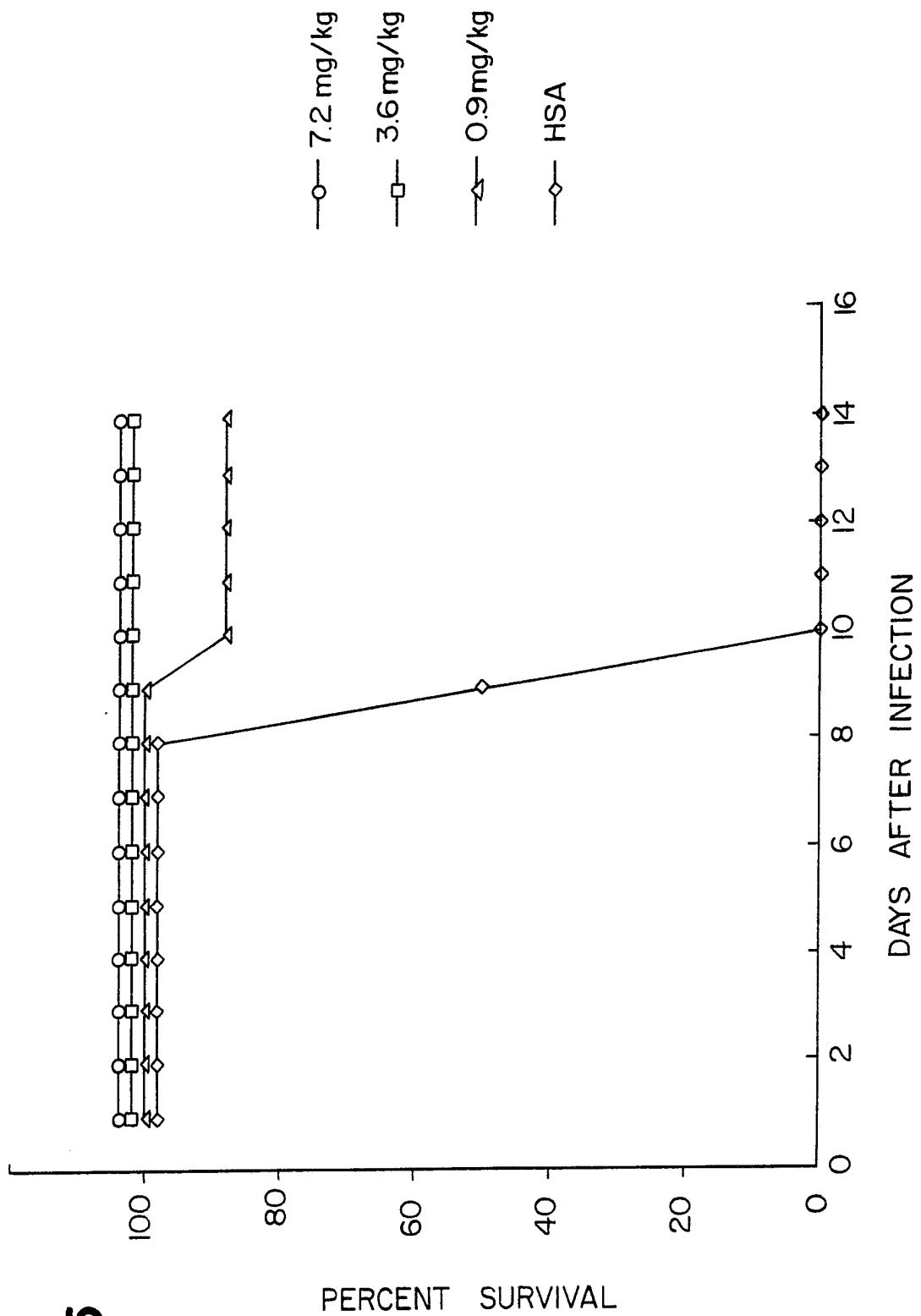
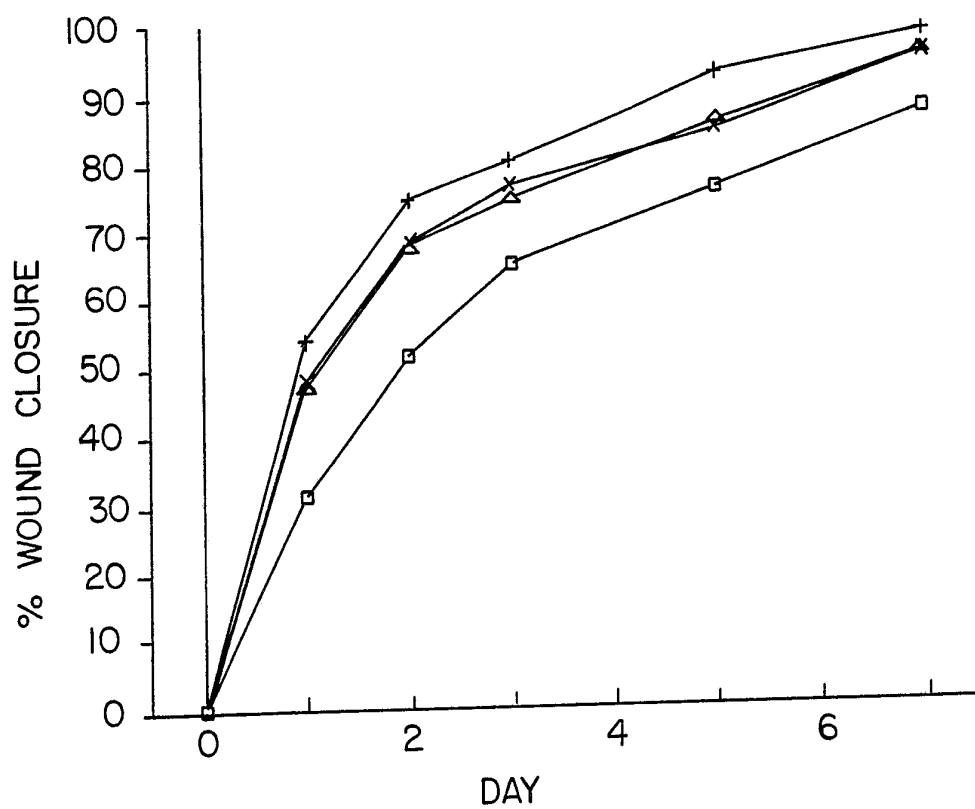


FIG. 5

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**FIG. 6**

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/06052

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 A 61 K 37/02 //(A 61 K 37/02 A 61 K 31:71) (A 61 K 37/02 A 61 K 31:41) (A 61 K 37/02 A 61 K 31:105) (A 61 K 37/02, 31:495) (A 61 K 37/02, 31:415)		
II. FIELD SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl.5	A 61 K	
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
X	The Journal of Immunology, volume 143, no. 2, 15 July 1989 (Baltimore, MD, US) M. Wang et al.: "Enhancement of human monocyte function against Candida albicans by the colony-stimulating factors (CSF): IL-3, granulocyte-macrophage-CSF and macrophage-CSF", pages 671-677, see pages 671-677 ---	1-3,8,9
X	The Journal of Immunology, volume 139, no. 2, 15 July 1987 (Baltimore, MD, US) A. Korbassi et al.: "Enhanced killing of Candida albicans by murine macrophages treated with macrophage colony-stimulating factor: evidence for augmented expression of mannose receptors", pages 417-421, see pages 417-421 --- -/-	1-3,8,9
¹⁰ Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "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) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "I" 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 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "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. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
18-12-1991	05. 02. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE		

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.
A	Biological Abstracts; volume 87, no. 11, 1989 (Philadelphia, PA, US) S. Oka et al.: "Clinical evaluation of Fluconazole", see page AB-205, abstract no. 112788, & Jpn. J. Antibiot. 42(1):31-3, 1989 ---	4,5
X	WO, A, 8902746 (CETUS CORPORATION) 6 April 1989, see the claims ---	14
P, X	Infection and Immunity, volume 59, no. 3, March 1991 (Washington, DC, US) E. Cenci et al.: "Macrophage colony stimulating factor in murine candidiasis: serum and tissue levels during infection and protective effect of exogenous administration", pages 868-872, see pages 868-872 -----	1-3, 8, 9

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers 6,7,10-13 because they relate to subject matter not required to be searched by this Authority, namely:

see PCT-Rule 39.1(iv).

Remark: Although claims 1-5,8,9,14 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

US 9106052
SA 51939

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82