Recombinant CSF's:

New Therapies for Granulocyte Disorders

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Medical Grand Rounds

University of Texas Health Science Center at Dallas

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Rx	Parkland Memorial Hospital
Ferrous Sulfate, 325 mg p.o. t.i.d. *Cyanocobalamin, 1000 µg i.m. q/month* *Folate, 1 mg p.o. qd* *Erythropoietin, 10,000 U IV q/3 days* ***GM-CSF, 100,000 U IV q3^{0} X 28 days***	
	R. Fleischman, M.D.

I. Introduction

The advent of recombinant DNA technology has created an exciting time for medicine and, in particular, for hematology and oncology. In the recent past, we have seen the introduction of interferon and interleukin-2 into medical practice. In the very near future, recombinant human erythropoietin, which has been shown to correct the anemia of renal failure, will be available for clincial use (Winearls et al, 1986; Eschbach et al, 1987).

Now entering clinical trials are another new class of substances termed "colony stimulating factors", or CSF's, because of their ability to stimulate the in vitro growth of colonies of hematopoietic cells (Sieff, 1987; Clark and Kamen, 1987). These substances promise to provide the clinician with a new ability to manipulate hematopoiesis and cell production. Moreover, they may profoundly alter the way in which we manage patients receiving chemotherapy and conceivably could be useful as supplemental treatments in severe infection.

TABLE I. Nomenclature of Human Colony Stimulating Factors

Factor Name	Other Names	Target Colony	Cell Lineage
G-CSF	CSF-β	CFU-G	Granulocytes
M-CSF	CSF-1	CFU-M	Macrophages
GM-CSF	CSF-α	CFU-GM	Gran. + Mono.
		CFU-Eo	Eosinophils
		CFU-Mega	Megakaryocytes
		BFU-E	Erythroblasts
		CFU-GEMM	Mixed
Multi-CSF	IL-3	CFU-GEMM	Mixed
		BFU-E	Erythroblasts
		CFU-GM	Gran. + Mono.
		CFU-G	Granulocytes
		CFU-M	Macrophages
		CFU-Eo	Eosinophils
		CFU-Mega	Megakaryocytes

Although nomenclature has historically been somewhat variable, these factors are generally named for the cell types that they stimulate in vitro (TABLE I). Thus, G-CSF stimulates a granulocyte precursor cell, which is itself called a CFU-G or colony forming unit (cell) for granulocytes. Similarly, M-CSF stimulates monocyte-macrophage precursors, and a cell that produces a colony of this lineage would be termed a CFU-M.

Two additional factors with more complex properties have also been described and recently cloned. These latter factors are characterized by the ability to stimulate the formation of colonies containing cells of more than one hematopoietic lineage. Thus, GM-CSF stimulates cells, termed CFU-GM, which are capable of producing colonies containing both granulocytes and monocyte-macrophages. Similarly, Multi-CSF (IL-3), stimulates the in vitro growth of colonies, some of which contain all the myeloid cell types, i.e. granulocytes, erythrocytes, macrophages, and megakaryocytes. The cell producing these latter colonies is therefore called a CFU-GEMM.

Finally, the factors of human origin are denoted by a small h before the factor name (i.e. hG-CSF). In addition, the recombinant form of the factor is indicated by a small r (i.e. rhG-CSF for recombinant human granulocyte colony stimulating factor).

II. Discovery of the CSF's

When investigators first took bone marrow cells and cultured them in liquid or semi-solid media, growth was very short-lived or non-existent (for a review, see Metcalf, 1977).

If a mouse was inoculated with endotoxin, however, and the serum collected several hours later was then mixed with the culture medium, large numbers of colonies were shown to develop. These colonies contained granulocytes, macrophages, or in some cases both cell types. The proportions of the different colonies obtained in fact varied somewhat with the amount of stimulator. FIGURE 1 is a schematic representation of the results one observes in such an experiment.

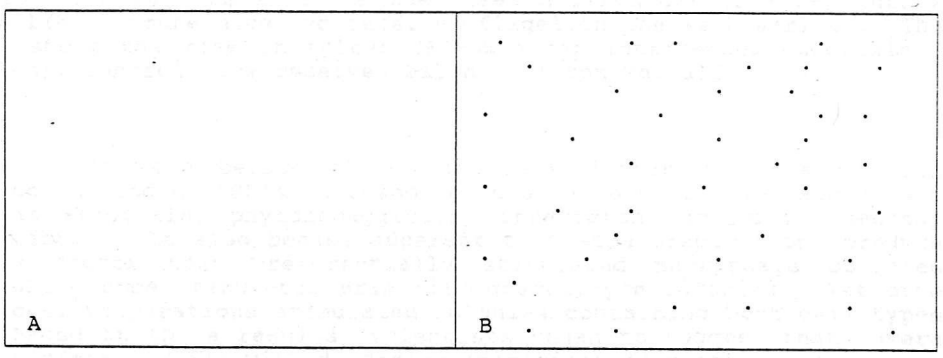

Fig. 1 Results of 7 day bone marrow culture without endotoxin serum (A), and with addition of endotoxin serum (B).

Over a period of hours, the level of this stimulator in the mouse's serum was observed to rise to a high level and then fall back to normal in a biphasic curve, as shown in FIGURE 2. Similarly, the number of progenitor cells for granulocytes and macrophages, the CFU-GM, also rapidly increased in the spleen, reaching a peak at four days after the injection of endotoxin. by two weeks, the levels of these progenitor cells returned to normal.

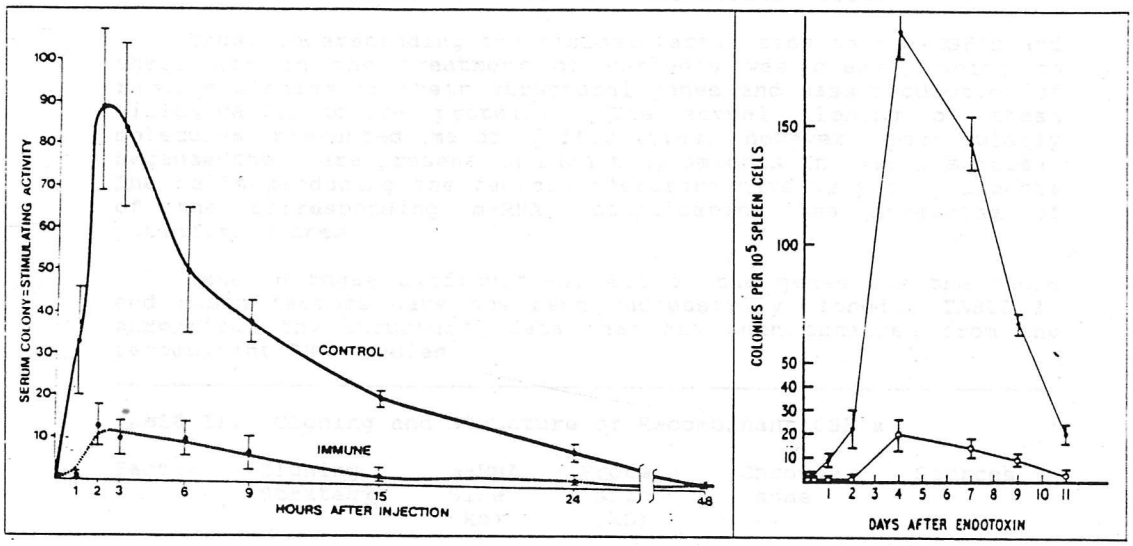

Fig. 2 The left panel shows the rise in serum GM-CSF after intravenous flagellin. Immune mice had received flagellin one week earlier. The right panel shows the rise in spleen CFU-GM after intravenous endotoxin (closed circles). Control mice received saline. (From Metcalf, 1977).

It soon became clear that many different kinds of cells could produce CSF's in response to a variety of stimulators, such as endotoxin, phytohemagglutin, interferon, and tumor necrosis factor. It also became apparent that some preparations produced a factor that preferentially stimulated macrophage colonies, while some stimulated primarily granulocyte colonies. Yet other cell preparations stimulated colonies containing both cell types. Based on these results, scientists began to suggest that several different CSF's with differing specificities might exist.

III. Cloning and Structure of Recombinant CSF's

Although the CSF's proved to be very potent, exerting their effects at concentrations of 10^{-11} to 10^{-13}, even the richest tissue sources of these molecules produced only minute amounts. Moreover, the half-life of these factors in vivo was short, probably minutes to hours. For these reasons, the early studies of the CSF's were plagued by problems of purity. Contamination with endotoxin or more than one CSF could obviously produce unexpected effects by stimulating small numbers of accessory cells that are present even in partially purified bone marrow cells.

Thus, understanding the biologic activities of the CSF's and their use in the treatment of patients was clearly going to require cloning of their structural genes and mass-production of biologically active protein. The actual cloning of these molecules presented major difficulties, however, particularly because they were present in such tiny amounts in tissue samples. The cells producing the factors therefore have very tiny amounts of the corresponding m-RNA, complicating the screening of potential clones.

Despite these difficulties, all of the genes for the mouse and human factors have now been successfully cloned. TABLE II summarized the structural data that has been obtained from the recombinant DNA studies.

TABLE II. Cloning and Structure of Recombinant CSF's

Factor	Cloning Strategy	m-RNA Size (kb)	Protein size (kD)	Chromosome	Sources
G-CSF	Sequencing	2.0	18-22	17p	Monocytes Fibroblasts
M-CSF	Sequencing	1.8 4.0	18-26 35-45	5q	Monocytes Fibroblasts Endothelium
GM-CSF	Direct Expression	1.0	14-35	5q	T-cells Endothelium Fibroblasts
Multi-CSF	Direct Expression	1.0	14-28	5q	T-cells

A. G-CSF and M-CSF

Two different strategies were used for successfully cloning the CSF structural genes. The first of these approaches relies upon a successful purification of the factor from natural sources in sufficient quantities to obtain a partial amino acid sequence. From this data, a specific oligonucleotide probe coding for these amino acids is then synthesized and used to screen a cDNA library.

Using this approach, the gene encoding hG-CSF has been cloned (Nagata et al, 1986a; Souza et al, 1986). Starting with a human bladder carcinoma cell line and a squamous carcinoman cell line, both of which produce large amounts of hG-CSF, the factor was first purified to homogeneity. After establishing a partial amino acid sequence, a synthetic oligonucleotide encoding for the sequence was synthesized. Finally, this probe was used to screen a cDNA libraries constructed from the m-RNA's produced by the original cell line.

Fortunately, one c-DNA clone was obtained that coded for a protein of 207 aa which was subsequently shown to have G-CSF activity equivalent to that of the native molecule. Of the 207 amino acids, only 177 actually comprise the mature protein; the other 30 appear to be part of a leader sequence.

The structural DNA sequence coding for the m-RNA of hG-CSF has recently been isolated (Nagata et al, 1986b). The gene itself has five exons and four introns. An alternative splicing site in intron 2 leads to the production of two different m-RNA's for hG-CSF. It is not yet known if there is a significant difference in these two proteins, although both are biologically active.

An analogous strategy was used for M-CSF. The protein was first purified to homogeneity from human urine (Stanley et al, 1975). Oligonucleotide probes from the amino terminal sequence of the hM-CSF were then used to isolate several clones from a human genomic library (Kawasaki et al, 1985). These clones were screened on a cDNA library made from the mRNA of a pancreatic tumor cell line that constitutively produced M-CSF. One of these clones, tested in a transient expression assay by introduction in monkey cells, was found to produce a substance with M-CSF.

It was subsequently shown that two different size m-RNAs are also produced for hM-CSF (Kawasaki et al, 1985; Wong et al, 1987). A 1.8 kb m-RNA produces a 16 kD precursor that is extensively N-glucosylated to yield a glycoprotein of 20-25 kD. The native molecule consists of a dimer of 40-50 kD. The 4.0 kb m-RNA, which is more abundant, produces a 61 kD precursor. However, the large protein is first processed to a 21-26kD protein, then N-glycosylated to 35-45 kD, and finally dimerized to yield a 70-90 kD form of hM-CSF.

B. GM-CSF and Multi-CSF

These latter factors were cloned by a different approach which avoids the need to purifiy the protein and perform amino acid sequencing. However, this strategy requires a highly sensitive bioassay to screen pools of many clones for the production of factor, and then, by progressive elimination, isolate the single cDNA responsible for the synthesis of the factor.

Thus, a cDNA library was first constructed from the m-RNA of a cell line that constitutively produced large amounts GM-CSF (the Mo T-lymphoblast cell line infected with HIV II) (Wong et al, 1985). Then random pools of the many cDNA clones were tested for their ability to transiently express hGM-CSF activity after introduction into mammalian cells, the monkey COS-1 cells. Although hGM-CSF is present in picomolar quantities, its high specific activity permitted its detection even in the minute quantities produced by the transfected cells.

By progressive elimination using smaller and smaller cDNA pools, a single cDNA of 432 nucleotides was isolated. The cDNA encoded a 144 amino acid precursor protein which was processed by cleavage of 17 amino acids from the amino terminal sequence to yield mature protein of 127 aa. The genomic clone is composed of four exons and three intervening sequence (Miyatake et al, 1985a).

Both natural and recombinant human GM-CSF yield identical glycoproteins of molecular mass of 14-35 D, the variability probably due to variable glycosylation. The human protein is 60% homologous to mouse GM-CSF, but there is no cross-species activity (Metcalf et al, 1986a).

Although murine IL-3 or Multi-CSF was cloned several years ago by a similar approach (Yokota et al, 1984; Fung et al, 1984), until recently the human analog proved more difficult to identify. The breakthrough came, however, when the CSF activity of a gibbon T-cell line, known to produce GM-CSF, was found to be only partially inhibited by antisera to GM-CSF (Yang et al, 1986). This result suggested the presence of another factor, i.e. Multi-CSF. Pools of cDNAs from this cell line were analyzed therefore by the direct expression screening method used for GM-CSF. However, in this case the cDNA pools were screened for the production of an activity that was resistant to neutralization by the anti-GM-CSF. In this manner, a cDNA was isolated which proved to encode a CSF with low but significant homology to mouse Multi-CSF. Using this clone as a probe, it has proved to be relatively easy to identify the corresponding human gene sequence.

Both the human and murine genes have 5 small exons separated by one large and three small introns (Yang et al, 1986; Miyatake et al, 1985b). At the nucleotide level there is 49% homology

between the two genes. The protein encoded by the 1.0 kb m-RNA is 14-28kD after glycosylation and the mature protein contains 177 aa (18.7 kD).

Although GM-CSF and Multi-CSF are similar in size, post-translational modification, numbers of glycosylation sites, and, most strikingly, in their biologic activity and production by activated T cells, there is absolutely no sequence homology between these two molecules.

IV. In Vitro Activity of CSF's

As mentioned briefly in the Introduction, each CSF is named for the hematopoietic cell lineages it stimulates in culture. With the introduction of the recombinant molecules, a more precise description of the activities of these factors has become possible. Previous problems with contamination of "partially purified" factors, particularly with other CSF's and cytokines, have largely been eliminated. FIGURE 3 summarizes the known interactions of the CSF's on the hematopoietic cell types and also illustrates the lineage tree of hematopoietic development.

A significant problem that persists in this work, however, concerns the purity of the hematopoietic cells. It is still not possible to be sure that an observed effect of a CSF represents a direct effect on the hematopoietic cell, or alternatively an indirect effect due to the secretion of other factors by contaminating cells of other lineages. The increasing use of partially purified preparations of hematopoietic progenitors, plated at very low densities, has in recent years partially corrected this technical difficulty.

A. G-CSF and M-CSF

The spectrum of activity of these two factors is apparently limited to essentially one cell type.

Human G-CSF appears in the most recent experiments with recombinant or highly purified native factor to stimulate predominantly granulocyte colonies (Nagata et al, 1986a; Souza et al, 1986). The human rG-CSF is nearly completely cross-reactive with the mouse G-CSF in biological assays and in receptor binding studies (Nicola et al, 1985).

In addition, recombinant hG-CSF stimulates mature granulocytes to an activated state, as judged by assays of superoxide generation, phagocytic ability, and antibody-mediated killing of tumor cells (Platzer et al, 1985, 1987; Lopez et al, 1986). Both human and mouse G-CSF also stimulate the terminal differentiation of some granulocytic leukemia cells, but in other cases stimulate progressive growth (Sabatini et al, 1982; Nicola et al, 1983; Griffin and Lowenberg, 1986; Griffin et al, 1986).

Human M-CSF similarly induces the formation of predominantly

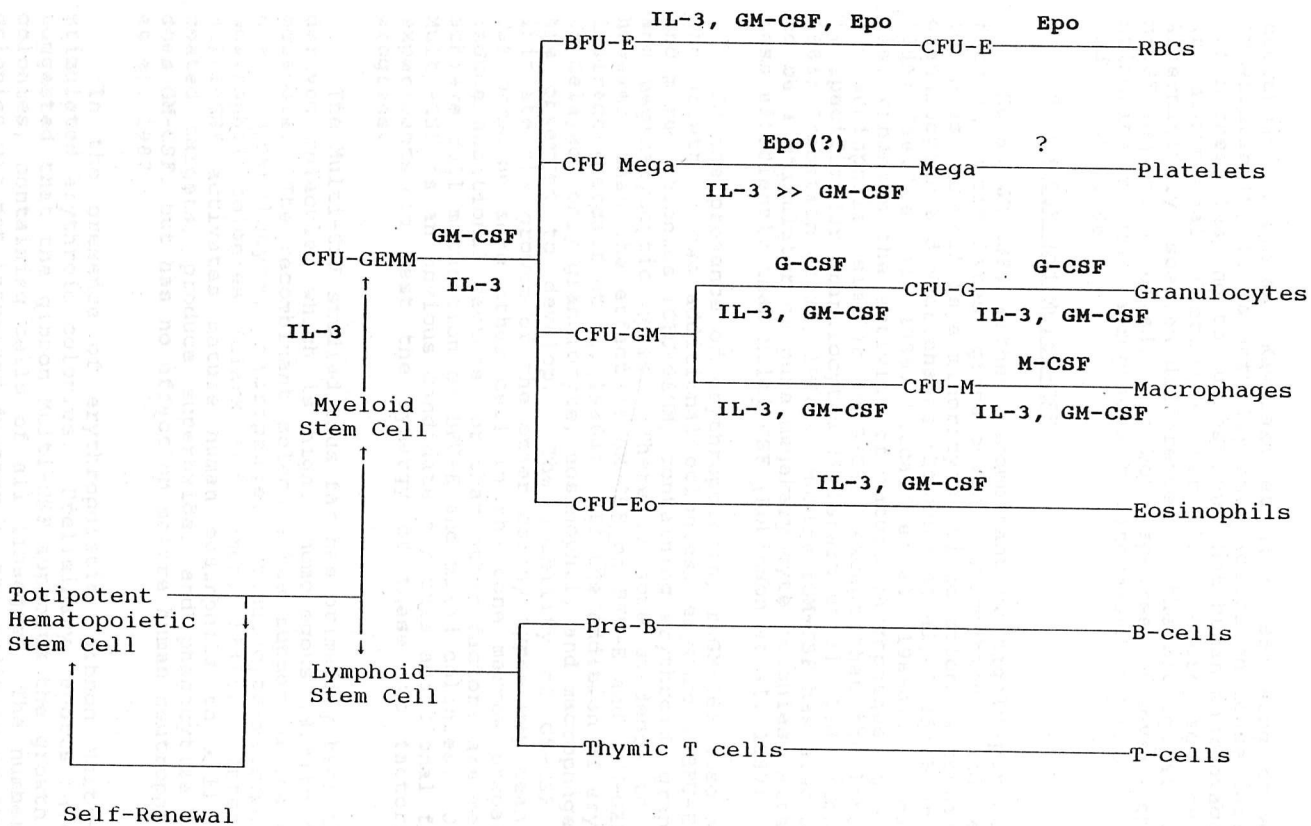

Figure 3. Interactions of CSF's with the pathways of hematopoiesis.

macrophage colonies (Kawasaki et al, 1985; Wong et al, 1987). Interestingly, it is actually more active on mouse cells than on human ones, leading to suggestions that human macrophages require an additional factor(s) for full macrophage development. Alternatively, species differences in the biological function of M-CSF may be involved. In both species, however, the M-CSF's stimulate mature macrophages to increased cytotoxicity (Warren and Ralph, 1986).

B. GM-CSF and Multi-CSF

Human GM-CSF, either recombinant or highly purified native factor, stimulates granulocyte, macrophage, and eosinophil colonies, as well as a minority of mixed colonies containing both granulocytes and macrophages (Donahue et al, 1985; Emerson et al, 1985; Sieff et al, 1985; Metcalf et al, 1986a). Human rGM-CSF also enhances the activity of mature macrophages, granulocytes, and eosinphils, similar to G-CSF, except that the latter factor is specific for granulocytes (Weisbart et al, 1985; Lopez et al, 1986; Grabstein et al, 1986). Murine rGM-CSF has also been shown to be a stimulator of pure megakaryocyte colonies, although much less efficiently than Multi-CSF (Robinson et al, 1987).

In the presence of erythropoietin, rhGM-CSF also stimulates the growth of two additional colonies, erythroid BFU-E colonies and mixed colonies (CFU-GEMM) containing erythroid, granulocytic, and megakaryocytic cells. There is some evidence to indicate, however, that the effect of GM-CSF on BFU-E and CFU-GEMM may be indirect (Metcalf et al, 1986a). If the addition of eryropoietin is delayed, only granulocyte, eosinophil, and macrophage colonies are observed to develop. The inability of GM-CSF alone to initiate the growth of the other colony types may mean that GM-CSF acts on some other cell in the bone marrow preparation to induce additional factors, or that other factors are required to achieve full maturation of BFU-E and mixed colonies. Of course, Multi-CSF is an obvious candidate for this additional factor and experiments to test the synergy of these two factors are in progress.

The Multi-CSF studied thus far has primarily been the gibbon derived molecule, which is highly homologous (93%)to the human molecule. The recombinant molecule has supported the growth of human granulocyte, macrophage, granulocyte-macrophage, and eosinophil colonies (Clark and Kamen, 1987). Interestingly, Multi-CSF activates mature human eosinphils to kill antibody-coated targets, produce superoxide, and phagocytize yeast, as does GM-CSF, but has no effect on mature human neutrophils (Lopez et al, 1987).

In the presence of erythropoietin, gibbon Multi-CSF also stimulated erythroid colonies. Preliminary reports have further suggested that the gibbon Multi-CSF supports the growth of mixed colonies, containing cells of all lineages. The number of such colonies was not increased further by the addition of GM-CSF. In

other unpublished experiments, the Multi-CSF appears to be much more potent than GM-CSF in supporting the growth of an even more primitive colony type, termed 21-day blast-cell colonies (Clark and Kamen, 1987). In addition, the colonies produced by Multi-CSF were capable of higher efficiency in replating, suggesting that Multi-CSF does indeed stimulate an earlier progenitor cell that GM-CSF.

V. Regulation of CSF Gene Expression

For many years, experimental hematologists have argued about the physiologic role of the colony-stimulating factors. At one extreme have been those who suggested that the factors had no relevance to in vivo hematopoiesis. These critics argued that the CSF's were merely molecules necessary for the in vitro growth of these cells, but either were not required in vivo or were already present in vivo in adequate amounts.

At the other end of the debate were those, including the investigators working on purifying these substances, who argued that these molecules were essential for granulopoiesis and hematopoiesis in vivo. Their concept was that the in vivo role of the CSF's in granulopoiesis would prove to be analogous to that of erythropoietin in erythropoiesis. Thus, a decrease in the production of the factor in vivo would lead to a decrease in the production of myeloid cells.

Although this argument has not yet been completely settled, a consensus has begun to emerge that the CSF's are almost certainly important emergency stimulators of granulopoiesis and myelopoiesis in vivo, particularly in response to infection. On the other hand, the data for their role in regulating normal homeostatic levels of cell production has been essentially negative to date, although additional experiments may change this conclusion.

The evidence against the CSF's as regulators of normal homeostasis in vivo consists of the following data:

(1) Although endotoxin, irradiation, anti-neutrophil serum, and cyclophosphamide lead to a rise in serum CSF levels in mice (Metcalf, 1971; Morley et al, 1971; Shadduck and Nagabhushanam, 1971; Shadduck and Nunna, 1971), germ-free mice demonstrated no rise in levels after irradiation (Morley et al, 1972).

(2) No increases in serum or urine levels of GM-CSF could be observed in patients with chronic neutropenia of more than 1 year's duration (Wewerka and Dale, 1976), or in children with severe congenital neutropenia (Amato et al, 1976).

(3) Injection of an anti-CSF preparation into mice did not lead to reductions of blood granulocytes, monocytes, or bone marrow precursor cells despite sustained levels of anti-CSF in

the blood (Boegel et al, 1985).

(4) Using the newly available DNA probes for the CSF's, to date it has not been possible to detect a level of constitutive m-RNA synthesis for either G-CSF, GM-CSF, or Multi-CSF in normal unstimulated cells. The cells examined include monocytes, fibroblasts, endothelial cells, and T cells (Sieff et al, 1987; Broudy et al, 1986; Munker et al, 1986; Sieff, 1987; Clark and Kamen, 1987).

On the other hand, injections of partially purified preparations of native CSF's have been shown to induce a granulocytosis in vivo (Metcalf and Stanley, 1971). This data has now been confirmed with the recombinant materials (see section VI).

In addition, cells stimulated by a variety of inducers synthesize readily detectable levels of m-RNAs for G-CSF, GM-CSF, and Multi-CSF. Thus, after exposure to endotoxin, gamma-interferon, or phorbol esters, monocytes are induced to synthesize high levels of mRNA for G-CSF and M-CSF, but not GM-CSF (Ramaldi et al, 1987; Sieff, 1987). In addition, IL-1 and TNF, which can be produced by stimulated monocytes, appear to induce the expression of GM-CSF and G-CSF by endothelial cells and fibroblasts (Zucali et al, 1986, 1987; Bagby et al, 1986; Fibbe et al, 1986; Broudy et al, 1986; Munker et al, 1986, Sieff et al, 1987). Activated T cells express GM-CSF and IL-3 mRNA, but not G-CSF or M-CSF mRNA (Yang et al, 1986; Wong et al, 1987; Ramaldi et al, 1987).

While the interactions of the cytokines and the cells producing them is still not fully understood, FIGURE 4 shows a schematic diagram of the probable relationships of these cells and their activation by the different factors.

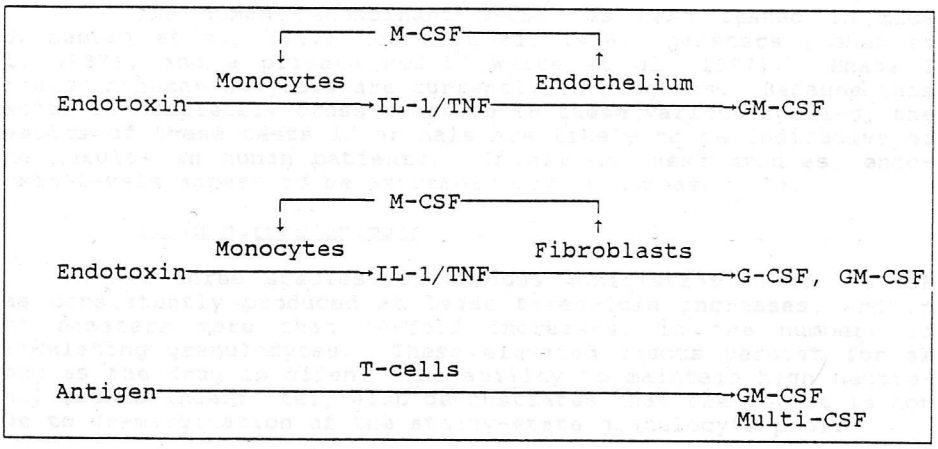

Fig. 4.

Overall, the data is consistent with the idea that CSF's are produced in response to infection, and are involved in emergency responses, but that their role in maintaining normal production of these cells types is still speculative. It is possible, of course, that extremely low basal levels of these factors are produced to maintain cell production in the unstimulated state. Presumably the stromal cells of the bone marrow, such as endothelial cells or fibroblasts, would be the source of these activities. Some recent evidence has suggested that GM-CSF is present in bone marrow cultures bound to glycosaminoglycans (Greaves et al, 1987).

M-CSF is an exception to the above data. The m-RNA for M-CSF is readily detectable in a variety of unstimulated tissues and cells (Kawasaki et al, 1985; Wong et al, 1987). The reason for the constitutive expression of M-CSF is not clear, but it has been suggested that it may be necessary to promote long-term survival of macrophages in the tissues.

VI. In Vivo Testing of the CSF's

The recent demonstrations, described below, of the in vivo effects of the CSF's has generated considerable excitement, both among hematologists, as well as among the stockholders of several biotechnology companies. In interpreting all of these studies, however, it is important to remember that the effects may be a direct action of the factor on on the hematopoietic target cell, but may also be an indirect effect due to the release of other factors.

A. G-CSF (Amgen Corp.)

The human recombinant G-CSF has been tested in mice (Shimamura et al, 1987; Moore et al, 1986), hamsters (Cohen et al, 1987), and a primate model (Welte et al, 1987). Phase I trials in human subjects are currently in progress. Because this factor is completely cross-reactive in these various species, the results of these tests in animals are likely to be indicative of the results in human patients. In all of these studies, endotoxin levels appear to be extremely low or unmeasurable.

1. In normal animals

In all three species, continuous administration of rhG-CSF has consistently produced at least three-fold increases, and in the hamsters more than 10-fold increases, in the numbers of circulating granulocytes. These elevated counts persist for as long as the drug is given. The ability to maintain high neutrophil counts indefinitely also demonstrates that the effect is not due to de-margination of the steady-state granulocyte pool.

In addition, the circulating granulocytes in these animals

were functionally active and appeared to have an enhanced ability to kill phagocytized bacteria, as judged by a doubling of nitroblue tetrazolium reduction. Parallel increases in the total number and in the number of cycling CFU-G in the bone marrow and/or spleens of these animals were also seen. Indeed, in one mouse study reported in abstract form, continued administration of G-CSF eventually led to splenomegaly (Moore et al, 1986).

In accord with the in vitro data, the rhG-CSF did not increase the numbers of monocytes, eosinophils, or reticulocytes. Interestingly, there was a 2-3 fold increase in the numbers of circulating T cells in the monkeys, but not in the rodents. Platelet counts were somewhat depressed, however, in the hamsters treated with higher doses.

2. In neutropenic animals

Following neutropenia induced by cyclophosphamide or 5-fluorouracil, the recovery of peripheral neutrophil counts was significantly accelerated in all three models, as shown in FIGURE 5. In the primate study, the bone marrow cellularity was also improved dramatically when examined two weeks after the cytotoxic drug. Pretreatment with the factor was not beneficial in the primates, however, and may have resulted in a slightly more severe, although brief, nadir.

An unusual aspect of this primate model, as compared to clincial experience in patients, is the very slow recovery of granulocyte counts observed in the controls, requiring nearly four weeks to return to thel normal range. In contrast, the kinetics of platelet recovery, requiring approximately 2 weeks to return to normal, was more typical of that seen in patients treated with these drugs. While platelet recovery was reported to be similar in both treated and untreated monkeys, in the hamster study platelet recovery after either 5-FU or cyclophosphamide was actually slightly delayed.

In one of the mouse studies (Shimamura et al, 1987), careful examination of granulocyte progenitor recovery revealed two distinct phases. The first occurred in the bone marrow beginning about 1 week after 5-FU, and the second occurred in the spleen approximately 2 days later. rhG-CSF had relatively little effect on the bone marrow recovery, but did dramatically stimulate the recovery in the spleen. Similarly, in a model for bone marrow transplantation, while G-CSF produced 4-12 fold increases in progenitors and 3 fold increases in mixed colony cells 2 weeks after lethal radiation and bone marrow transplantation, these increases were predominantly in the spleen (Moore et al, 1986). This pattern of recovery, bone marrow preceding spleen, is consistent with the generally accepted evidence that the stem cell and self-renewal activity of the hematopoietic system resides primarily in the bone marrow.

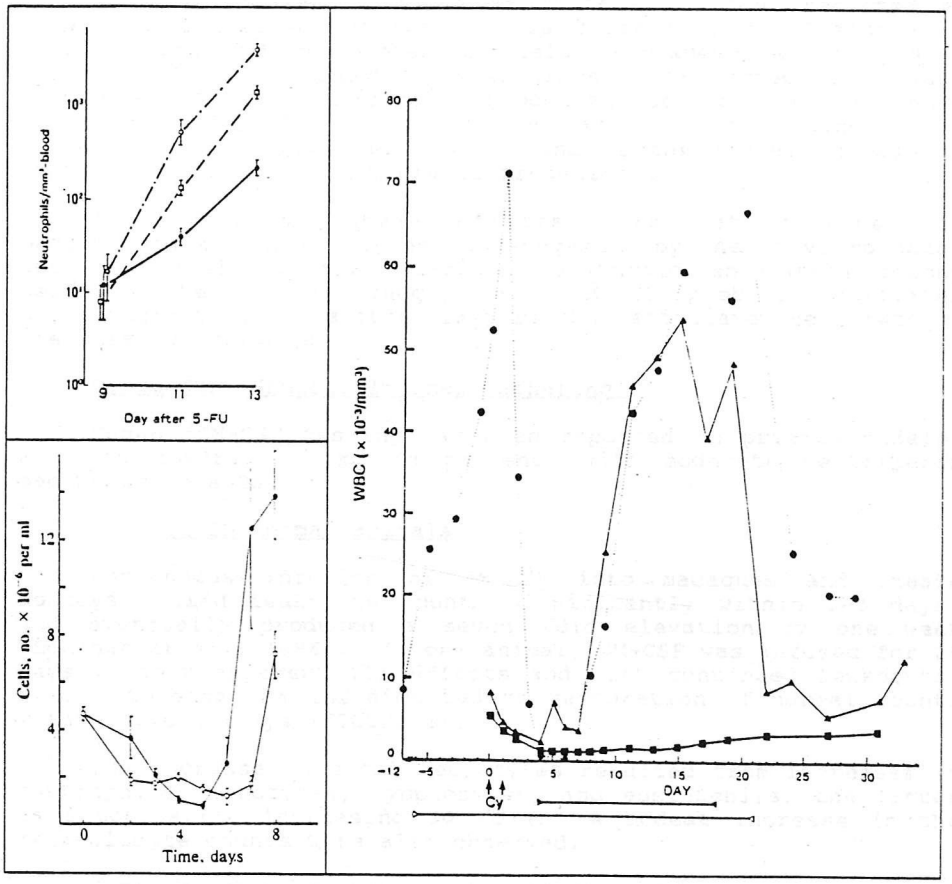

Fig. 5. Recovery from neutropenia in animals treated with rhG-CSF

The upper left panel shows granulocyte counts in control (solid line) and experimental mice (dashed lines) from 9-13 days after 5-FU (Shimamure et al, 1987). The lower left panel depicts the leukocyte counts in control (open circles) and experimental hamsters (closed circles) after treatment with 5-FU (Cohen et al, 1987). The right panel shows the leukocyte counts in a control (squares), GM-CSF pre-treated (circles), and GM-CSF treated monkeys (triangles) after cytoxan (Welte et al, 1987).

B. M-CSF

In vivo studies of human M-CSF have not been reported to date. In the mouse, however, it is clear that M-CSF also is a strong stimulator of progenitor cells (Broxmeyer et al 1987a, 1987b, 1987c). In short term experiments performed in animals pretreated with an inhibitor of progenitor cell cycling, a single injection of M-CSF produced increases in the numbers of progenitors in active cell cycle, and at the highest doses, an increase in the total numbers of progenitors.

Interestingly, these effects were not confined to macrophages and granulocytes, as suggested by the in vitro data, but also included enhancement of erythroid and mixed colony cells. The authors suggest that M-CSF probably activates macrophages to release other factors that stimulate the growth of these latter colonies.

C. GM-CSF (Sandoz, Immunex, Schering)

Human rGM-CSF testing has been reported in primate models, and, in abstract form, in patients with moderate neutropenia secondary to AIDS.

1. In normal animals

Continuous infusion of GM-CSF into macaques and rhesus monkeys raised leukocyte counts significantly within 1-3 days, and eventually produced a seven fold elevation by one week (Donahue et al., 1986). In one animal, GM-CSF was infused for 28 days without apparent ill effects and with continued leukocytosis. Stopping the infusion led to restoration of normal counts within several days (FIGURE 6).

The increase in total leukocytes resulted from increases in neutrophils, monocytes, lymphocytes, and eosinophils, the latter cell population increasing 30 fold. A modest increase in the reticulocyte counts were also observed.

Similar changes have also been reported in cynomolgus monkeys treated with recombinant human GM-CSF, the white blood cell count rising from a baseline of approximately 4,000 to 40-60,000 by 6 days (Welte et al, 1986). Once again, eosinophils acounted for 20-30% of the total leukocytosis, as compared to 0-1% of the baseline count in controls. Combined treatment with both G-CSF and GM-CSF produced a similar degree of leukocytosis, but, interestingly, without the eosinophilia.

2. In neutropenic primates

As shown in FIGURE 6, one severely pancytopenic rhesus monkey, thought to be infected with a simian type D retrovirus, was treated with a continuous infusion of GM-CSF for seven days

at a dose 10 times higher than that used for the normal animals. This treatment resulted in a rise in the white blood count from 1600 (98% lymphocytes, 2% monocytes) to 20-40,000 by 7-9 days (54% neutrophils, 42% lymphocytes). Following discontinuation of the drug, the white blood cell count returned to 1900. It would have been interesting to treat this animal subsequently with prednisone, another drug known to induce leukocytosis to compare the effects of these two agents. The effect of the treatment on the platelet counts are not described.

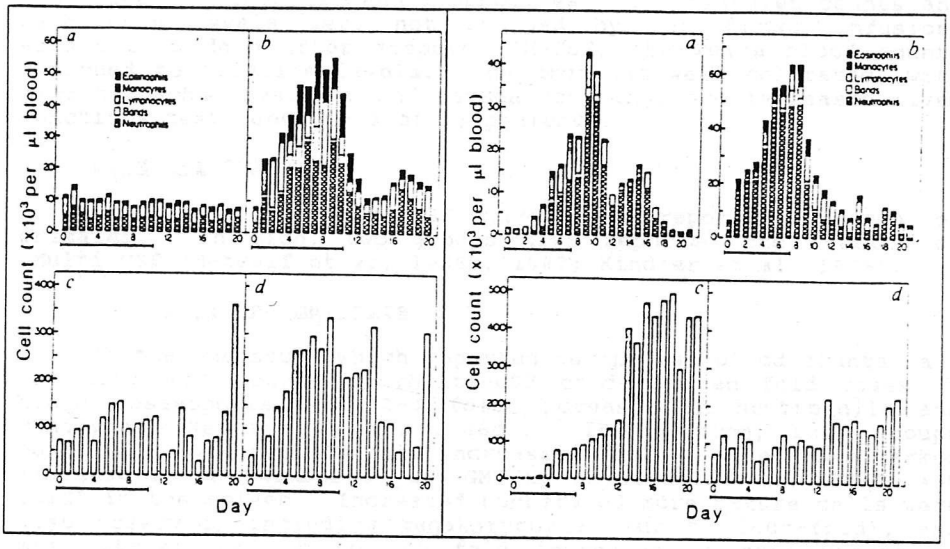

Figure 6. rhGM-CSF in normal and pancytopenic primates.

The left panel shows the the white blood cell (a,b) and reticulocyte (c,d) counts in a control macaque (a,c) and an experimental macaque (b,d). The right panel depicts a similar experiment performed in a pancytopenic rhesus monkey (a,c) and a normal control (b,d). (Donahue et al, 1986)

In another study, reported in abstract form, rhGM-CSF accelerated the recovery of granulocyte and platelet counts in rhesus monkeys undergoing autologous bone marrow transplantation. (Donahue et al, 1987). Discontinuing the factor, however, led to a prompt fall in the peripheral blood counts to the same levels observed in the corresponding controls. This results suggest that the observed effect resulted from stimulation of later progenitor cells in the graft. The stem cells of the bone marrow, which are capable of self-renewal and permanent repopulation, were apparently not affected by infusion of the factor.

3. In AIDS patients with neutropenia

Finally, in phase I trials, Groopman has reported preliminary results on the effect of rhGM-CSF in patients with neutropenia and AIDS (Groopman et al, 1987). At doses somewhat lower than those used in the primate studies, by 15-17 days rhGM-CSF elevated white blood counts from initial levels of 2000-3000 to 8,000-18,000. The increase in WBC counts was predominantly due to an increase in neutrophils and eosinophils, with mild increases in monocytes and lymphocytes. The platelet counts and hemoglobin levels were not changed by the factor infusion. Within 2-10 days after stopping GM-CSF, the white blood counts returned to baseline levels. The drug was well tolerated, with mild headache, myalgia, and nausea occuring, and increased liver functions tests seen in 3 of 16 patients.

D. Multi-CSF

No trials of Multi-CSF have been reported in man or primates. In mice, two groups have reported the results of rMulti-CSF (Metcalf et al, 1986b, 1987; Kindler et al, 1986).

1. In normal mice

In the one study which reported peripheral blood counts, a 6 day treatment course of rMulti-CSF produced ten fold rises in blood eosinophils, and 2-3 fold increases in neutrophils and monocytes (Metcalf et al, 1986b). In addition, both groups observed a two to three fold increase in spleen size and a marked increase in the number of CFU-GM (up to 30 fold) and CFU-E (4-8 fold) in the spleen. Increased numbers of more mature cells were also observed, including megakaryocytes (up to four-fold), and most strikingly, up to 100 fold increases in the numbers of splenic mast cells. Both groups also reported the presence of numerous hematopoietic foci in the livers of the treated animals, and one study also noted a prominent increase in the numbers of macrophages and eosinophils in peritoneal fluid (Metcalf et al., 1987).

In contrast, the cellularlity of the bone marrow was decreased aprroximately 20% in one study (Metcalf et al, 1986b), although the change was not statistically significant, and more than 50% in the other study (Kindler et al, 1986). In the latter study, the numbers of all progenitor cell types was also significantly reduced in the bone marrow after a 7 day infusion of rMulti-CSF. Overall, however, the authors calculated that there was at least a 2 fold increase in the total number of hematopoietic colony-forming cells.

2. In neutropenic mice

In one study, a 7 day infusion of rMulti-CSF was started immediately after sublethal irradiation (Kindler et al, 1986). At the end of the infusion, the spleens of the treated mice were more than 10 fold larger, splenic CFU-GM and CFU-E numbers were approximately 50-100 fold higher than those of the controls. On the other hand, the bone marrow of the treated mice was 20% less cellular, contained slightly fewer CFU-GM, and, strikingly, showed a 90% reduction in the number of CFU-E.

VII. Therapeutic application of the CSF's

Based on the animal and preliminary human studies described above, it is clear that rCSF's are capable not only of inducing a profound leukoctyosis but also of activating the host defense functions of mature cells. TABLE III summarizes the activities of G-CSF, GM-CSF, and Multi-CSF based on the in vivo data.

TABLE III. In Vivo activity of recombinant CSF's

Factor	Granulo-cytosis	Eosino-philia	Mature Cells Activated	Splenomegaly or EMH[1]
G-CSF	>10X	No	PMN's only	Yes
GM-CSF	7X	30X	Granulocytes Eosinophils Macrophages	?
Multi-CSF	2-3X	10X	Eo's only	Yes

[1]EMH - Extramedullary Hematopoiesis

The potential clinical applications of these agents therefore appear to fall into the following clinical situations. The potential benefit of CSF administration in rare congenital disorders of phagocytosis or neutrophil and macrophage function will not be considered here. For simplicity, I will also only discuss the uses of single agents. Combinations of these factors, of course, may eventually prove to be more efficacious than any one factor alone.

A. In severe infection

Although CSF's probably function primarily as growth factors, they all clearly have an additional capacity to stimulate the phagocytic functions of granulocytes, macrophages, and/or eosinophils. Since granulocytes play a vital role in host defense against bacterial infections, G-CSF and GM-CSF could conceivably be useful adjuncts to antibiotic therapy for the treatment of severe infections. Similarly, M-CSF and GM-CSF stimulate macrophage activity and may thus be helpful in infections involving predominantly intracellular bacteria (Mycobacteria, Listeria, Salmonella, Legionella, Brucella, Chlamydia, Rickettsia) and protozoa (Leishmania, Toxoplasma, Trypanosoma). Stimulation of eosinophil function with GM-CSF may also be of benefit in patients with certain parasitic diseases.

One difficulty with this logic, however, is that CSF levels in patients with these infections should already be highly elevated as a natural response to the foreign organism. One can speculate, on the other hand, that debilitated patients or patients with malignancy or other serious medical problems may respond to infection with less than maximal levels of CSF's. In addition, one can not rule out the posssibility that a further increment in granulocyte numbers and activity can be achieved with pharmacologic, rather than physiologic, doses of CSF's.

It should also be noted that partially purified hGM-CSF actually inhibits the migration of neutrophils and decreases their adherence to endothelial cell surfaces (Gasson et al, 1984; Lopez et al, 1986). Although these would be useful actions for locally released GM-CSF, presumably to retain neutrophils at the site of infection, systemic administration of these factors might actually decrease ingress of phagocytic cells into a bacterial abscess, for example. Possibly, local injections of CSF's may be feasible in some situations. Clearly, careful clinical trials will be required to demonstrate the efficacy of CSF's in patients with severe infections and normal bone marrows.

B. In acute granulocytopenia

Perhaps the most widely discussed potential use for the CSF's is as stimulators of bone marrow recovery in patients with acute granulocytopenia secondary to aggressive chemotherapy. Patients recovering from immune-mediated drug induced granulocytopenias, which are characterized by accelerated granulocyte destruction, may also be candidates for this type of therapy. Of course, severely neutropenic patients are susceptible to life-threatening infections with bacteria and other organisms. Although anemia and thrombocytopenia can usually be treated effectively with transfusion, the extremely short half-life of granulocytes in the circulation has made it extremely difficult to correct neutropenia with transfusions.

From the data published thus far, it seems clear that in the most toxic drug regimens, treatment with CSF factors has not altered the time interval between cytotoxic therapy and the onset of the granulocyte nadir. Similarly, for the most toxic drug regimens the severity of the initial granulocytopenia seems to be unchanged. In addition, treatment with factor prior to cytotoxic therapy does not appear to be more effective than factor infusion commencing immediately after cytotoxic therapy. Indeed, on theoretical grounds, stimulation of progenitor cycling prior to cytotoxic therapy could actually increase the hematopoietic toxicity of cycle-specific chemotherapy.

Nevertheless, given the results of the animal studies, it seems very likely that continuous treatment with a CSF can indeed shorten the time interval between the nadir in granulocyte counts and the return to a normal granulocyte count. Moreover, this effect was achieved without a significant delay in the recovery of platelet counts. Despite this impressive data, several additional clinically relevant questions need to be answered:

(1) Will CSF treatment shorten the number of days required to recover to a granulocyte count that is sufficient to protect against infection? Although less than 1500 granulocytes per microliter is defined as abnormal, the risk of infection is not increased for counts in the 1000-1500 range, and increases only slightly for counts in the 500-1000 range (Williams et al, 1983). Thus, the critical time interval is likely to be the time to reach 500 absolute granulocytes per microliter. Acceleration of recovery after reaching 500 granulocytes may not therefore produce additional benefit.

(2) Will CSF administration benefit patients with established infection and severe neutropenia? As discussed previously, CSF levels should already be elevated in the presence of ongoing infection. Will an increase in these factors to more than physiologic levels translate into real clinical benefit? In addition, the data to date do not demonstrate that a higher number of granulocytes in the blood also leads to a higher number of granulocytes at the sites of infection.

(3) Will CSF treatment translate into an improvement in survival for chemotherapy and bone marrow transplant patients? A one or two day acceleration in granulocyte recovery to 500 per microliter may conceivably still be too small a difference to affect the liklihood of survival in these patients.

C. In chronic granulocytopenia

Another group of patients that may benefit from CSF's are patients with severe chronic granulocytopenia. Many of these patients have a myelodysplastic syndrome or aplastic anemia and suffer from recurrent infections, particularly if their granulocyte count is less than 500 per microliter. Moreover, in some of these patients, the granulocyte count actually rises to normal

or above normal during acute infectious episodes, presumably from the release of endogenous CSF's or other factors. Chronic administration of CSF's, then, may decrease the incidence of infection in patients with severe neutropenia, and particularly in those who have demonstrated at least a mild leukocytosis during prior infections.

Although isolated chronic granulocytopenia is occasionally observed in these disorders, most commonly deficiencies of other cell types are also present. rhGM-CSF and rhMulti-CSF, possibly in combination with erythropoietin, may prove to be useful in raising platelet counts and decreasing transfusion requirements in some of these cases.

It may also be possible to treat patients with milder degrees of granulocytopenia that limits the use of cytotoxic or anti-viral drug therapy. Examples of patients in this group would include those with neutropenia and 1) autoimmune diseases requiring immunosuppressive therapy, 2) malignancy requiring additional chemotherapy, or 3) AIDS requiring treatment with AZT or other cytotoxic drugs. Severe burn victims also commonly have low white blood cell counts.

Are there risks that may be associated with long-term administration of the CSF's? In the phase one trial conducted in AIDS patients, a 14 day infusion of rhGM-CSF was extremely well tolerated (Groopman et al, 1987). Of some concern is a recent meeting presentation showing that a monocyte cell line carrying a latent HIV virus in its genome was stimulated by rhGM-CSF to produce 3-4 fold more infectious HIV (Barnes, 1987). Only rGM-CSF caused this result; recombinant interleukin-1, interleu- kin-2, gamma interferon and tumor necrosis factor were inactive in this assay.

Another more general concern with long-term use of rGM-CSF and rMulti-CSF is the marked eosinophilia produced by these factors. The increased numbers of mast cells induced by Multi-CSF also could promote tissue eosinophilia through the release of histamine and eosinophil chemotactic factor. Although patients have tolerated eosinophilia for many years without ill effect, when eosinophil counts exceed 5000 per microliter, some authorties have recommended suppressive therapy with steroids or hydroxyurea (Williams et al, 1983). Tissue injury, due to substances released by disintegrating eosinophils, may otherwise develop in patients with the hypereosinophilic syndromes.

Finally, prominent splenomegaly and extramedullary hematopoiesis has occurred in mice treated with either rhG-CSF or rMulti-CSF. This effect may be species specific, as splenic enlargement was not seen in the hamster and primate studies. However, in monkeys treated with rhG-CSF for four weeks, the spleen, thymus,and lymph nodes showed foci of extramedullary hematopoiesis (Welte et al, 1987). No foci were observed in other organs and no toxicity was associated with these changes.

D. In leukemia

Although the CSF's can induce terminal differentiation in some primary leukemia cells and leukemia cell lines, they also act as growth factors for other leukemia cells (Griffin and Lowenberg, 1986). Moreover, a number of patients have been described whose blast cells constitutively produce GM-CSF (Griffin et al, 1986; Hoang et al, 1986). By an autocrine mechanism, the factor then acts to stimulate further growth of the leukemia cells (Young et al, 1987).

Clearly, administration of CSF's to patients with acute or chronic granuloctyic leukemias would therefore entail some risk. Nevertheless, some authors have advocated the use of the CSF's as differenitiation inducers in the therapy of both the myelodysplastic disorders and the granulocytic leukemias. In vitro testing of patients' cells with the CSF's may help in identifying those patients most likely to benefit from this approach.

Additional evidence suggests that the CSF's or their receptors may play a role in the evolution of some neoplasias. For example, it has been shown recently that the receptor for M-CSF is closely related to and possibly identical to the protein product of the c-fms protooncogene (Scher et al, 1985). This protein exhibits tyrosine kinase activity which is enhanced by binding of M-CSF. In contrast, v-fms, the oncogene of a feline sarcoma virus, is altered so that tyrosine kinase activity is constitutively expressed even in the absence of binding by the ligand, M-CSF (Coussens et al, 1986). Thus, introduction of the v-fms oncogene into a macrophage cell line converts it from M-CSF-dependent to factor-independent growth (Wheeler et al, 1986).

Finally it is interesting to note that the genes for M-CSF, the receptor for M-CSF, GM-CSF, and Multi-CSF are all located on the long arm of chromosome 5 (Pettenati et al, 1987; Le Beau et al, 1986; Huebner et al, 1985). (The structural gene for G-CSF is on chromosome 17). Deletions of this region of chromosome 5 [del(5)q] are observed in patients with myelodysplastic syndromes, acute leukemia secondary to previous cytotoxic therapy, and in a refractory anemia syndrome termed the 5q minus syndrome (Mitelman, 1985). The relationship, if any, of deletion of this gene cluster and the evolution of these disorders is not understood. However, it will be extremely interesting to see if patients who lack the distal end of chromosome 5 respond differently to treatment with GM-CSF and Multi-CSF than patients with other myelodysplastic and refractory anemia syndromes.

On the next page, TABLE IV summarizes some of the potential indications for CSF's in clinical practice and also lists some of the potential side-effects to these therapies.

TABLE IV. Indications for and Possible Complications of CSF's

Indication	Advantages	Possible Disadvantages
Infection	Leukocytosis	Thrombocytopenia (G-CSF)
	Activated PMN's	Decreased Migration
Acute ChemoRx Neutropenia	Accelerated Recovery	Decreased Stem Cell Self-Renewal in Bone Marrow
Chronic Neutropenia	Granulocytosis	Eosinophilia (Multi-CSF, GM-CSF)
	Decrease in Infections	Splenomegaly and Extra-meduallary Hematopoiesis
	Improved tolerance of immunosuppressive or antiviral therapy	Activation of latent virus infections (AIDS)
Leukemia	Differentiation of leukemic blasts	Stimulation of growth by clonogenic leukemia cells

VIII. Conclusion

The cloning of the genes for the human CSF's and the production of highly purified preparations of these proteins will allow hematologists to define in the next few years their therapeutic role in patients with bone marrow failure syndromes and hematologic malignancies. In addition, the availability of specific probes for the expression of these molecules will facilitate in situ hybridization studies to identify directly the important regulatory cells of the hematopoietic microenvironment. Finally, elucidation of the role of these factors and their receptors in myelodysplasia and leukemia should yield additional insights into the pathogenesis and treatment of these malignancies.

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