Articles

Control of Granulocytes and Macrophages: Molecular, Cellular, and Clinical Aspects

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The production and functional activity of two important white blood cells, the granulocytes and macrophages, are regulated mainly by a group of glycoprotein colony-stimulating factors. The colony-stimulating factors have been mass-produced with recombinant technology and are now proving of value in preventing or suppressing infections in a variety of individuals with subnormal or defective formation of blood cells.

TWO TYPES OF WHITE BLOOD CELLS, THE NEUTROPHILIC granulocytes and monocytes (or their tissue derivatives, the macrophages), are of importance in resistance to infections; abnormally low numbers of granulocytes are associated with susceptibility to serious infections (*1*). Like other blood cells, most granulocytes and monocyte-macrophages have short life-spans, and new cells must be produced continuously in adult life to maintain appropriate amounts of these cells. The regulatory problems posed in blood cell formation (hematopoiesis) are complex for several reasons. (i) Hematopoietic tissues are dispersed throughout the body in various bones, requiring mechanisms for coordinating cell production in multiple locations. (ii) All blood cells originate from a small common pool of multipotential hematopoietic stem cells, probably requiring mechanisms for achieving controlled commitment of progeny cells into the eight major blood cell lineages. (iii) Cell production in any one lineage requires numerous amplifying cell divisions coupled with complex maturation changes to produce the mature cells that are released into the blood (*2*).

Regulation of hematopoiesis is achieved by two interacting control systems. Specialized stromal cells in the marrow control some of the cellular events in hematopoiesis by cell contact processes or by the production of short-range regulatory molecules. This local stromal control seems to maintain stem cell numbers and the production by stem cells of progenitor cells committed to the formation of cells in a particular lineage. A second control system involves the coordinated interaction of a group of regulatory molecules (hematopoietic growth factors) that stimulate the proliferation of progenitor cells and their progeny and initiate the maturation events necessary to produce fully mature cells (*3*).

Colony-Stimulating Factors

Four distinct regulators, known collectively as the colony-stimulating factors (CSFs), are the dominant molecules controlling the production, maturation, and function of granulocytes and monocyte-macrophages (Fig. 1). They were discovered because of their ability to stimulate the formation of colonies of granulocytes and macrophages in semisolid cultures of bone marrow cells (*4*). The CSFs are produced by multiple cell types, including fibroblasts, endothelial cells, stromal cells, and lymphocytes, that are widely distributed throughout the body. The levels of CSF production are normally low, but production can be rapidly elevated in response to emergencies such as the occurrence of an infection (*3*).

The CSFs are glycoproteins with a varying content of carbohydrate and have molecular masses in the range of 18 to 90 kD (Table 1). Three of them [granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), and multipotential CSF or interleukin-3 (multi-CSF or IL-3)] consist of a single polypeptide chain, whereas the fourth, macrophage CSF (M-CSF), is a dimer of two identical subunits. Cysteine-cysteine disulfide bridges hold the molecules in a biologically active three-dimensional configuration, and multiple portions of the polypeptide chain contribute to the active binding domain. Peptide fragments of the CSFs have no biological activity, and because the CSFs are present in tissues in low concentrations, the only feasible method for producing sufficient CSFs for clinical use in vivo is by the generation of recombinant material. The carbohydrate portion of the CSFs is not required for biological activity in vitro or in vivo; thus, bacterial, yeast, or mammalian expression systems produce active, recombinant CSFs. For GM-CSF, G-CSF, and IL-3, each polypeptide contains a leader sequence that is cleaved before secretion (*5, 6*). For M-CSF, at least three alternative transcripts are produced, each of which permits M-CSF to be displayed on the membrane, where it can stimulate target cells after cell contact (*7*). Soluble M-CSF can be released by proteolytic

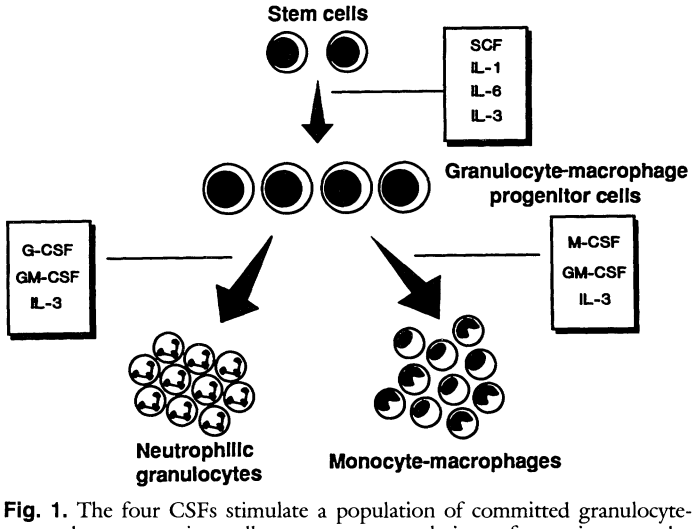

Fig. 1. The four CSFs stimulate a population of committed granulocyte-macrophage progenitor cells to generate populations of maturing granulocytes or monocyte-macrophages. Several growth factors can influence the formation of progenitor cells by some cells in the more ancestral hematopoietic stem cell compartment.

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Table 1. The human CSFs (*10*).

Acronym	Polypeptide chain (kD)	Native glycosylated (kD)	Chromosomal location
GM-CSF	14.7	18–30	5q23–31
G-CSF	18.6	20	17q11.2–21
M-CSF	21, 18	45–90	1p13–21
IL-3	15.4	15–30	5q23–31

cleavage of these membrane-bound forms.

Each CSF can initiate the proliferation of responding cells by forcing noncycling cells into the mitotic cycle. The concentration of CSF then determines the length of the cell cycle and the total number of progeny produced. Although each CSF can stimulate cell proliferation at low concentrations (Fig. 2), the CSFs differ in the pattern of proliferation they elicit. In vitro, both GM-CSF and IL-3 stimulate the formation of granulocytic and macrophage colonies, whereas G-CSF tends to be a selective stimulus for granulocyte colony formation, and M-CSF is a relatively selective stimulus for macrophage colony formation (*3*).

The CSFs are not simply proliferative stimuli but are polyfunctional regulators. The CSFs also control differentiation commitment in granulocyte-macrophage progenitor cells, the initiation of maturation, and the functional activity of the mature cells finally produced (*3, 8*). The latter action involves a variety of functions, including the maintenance of membrane transport systems, chemotaxis, phagocytosis, cytotoxicity, and the production and release by the cells of a number of biologically active molecules.

The common biological actions of the CSFs on granulocyte-macrophage populations led to the expectation that the CSFs would be a family of related regulators, but this was not supported by data from the amino acid sequences of the CSF polypeptides; the CSFs share no significant identity (*5, 6, 9*). However, other evidence has validated the concept that the CSFs are indeed a related family. (i) The CSF genes have common structures. (ii) The genes for GM-CSF and IL-3 are adjacent and are probably functionally linked on chromosome 5 in man (11 in the mouse). (iii) Three of the CSF receptors have sequence similarities.

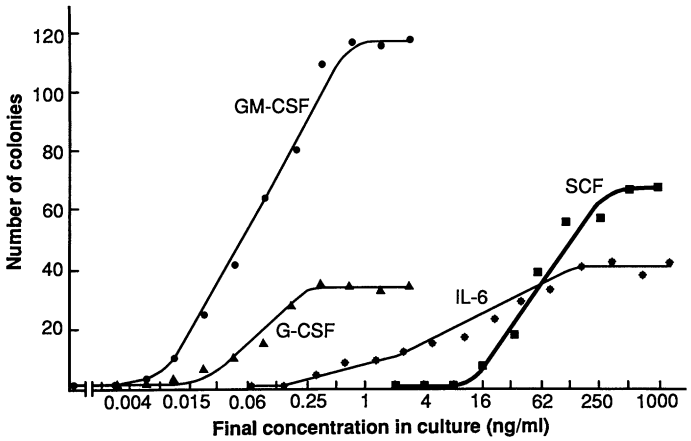

Fig. 2. Stimulation of the formation of granulocyte colonies in cultures of mouse bone marrow cells by G-CSF (*21*). GM-CSF (*21*) stimulates the formation of both granulocyte and macrophage colonies in these cultures. IL-6 (*20*) and SCF (*21*) also stimulate granulocyte colony formation by murine cells, but higher concentrations are required. Figure adapted by permission from (*21*).

The Receptors

Most granulocyte-macrophage progenitors and their maturing progeny simultaneously express specific membrane receptors for all four CSFs, an arrangement allowing interactions between the CSFs on individual cells. Most cells have only a few hundred CSF receptors, but CSFs can elicit responses with occupancy of only a low percentage of these receptors (*10*). The M-CSF receptor is the product of the proto-oncogene c-*fms* and is a transmembrane glycoprotein with an intracytoplasmic tyrosine kinase domain similar in general structure to the platelet-derived growth factor receptor and the product of the proto-oncogene, c-*kit* (*11*). Although the receptors for the other three CSFs are also transmembrane glycoproteins, they lack a tyrosine kinase domain and must elicit signaling by other mechanisms. These three CSF receptors exhibit homology in their extracellular domains, characterized by matching cysteine residues and a common tryptophan-serine-X-tryptophan-serine motif (where X is any amino acid). These receptors also share homology with receptors for a number of other hematopoietic growth factors and are members of a newly recognized growth factor receptor superfamily (*12*). By analogy with the interleukin-2 (IL-2) and interleukin-6 (IL-6) receptors, it is presumed that each of the nontyrosine kinase CSF receptors may have at least one other subunit or associated polypeptide that is not necessarily able to bind CSF but does alter the affinity of receptor binding and help to initiate the signaling cascade. One of these receptor subunits for the human GM-CSF receptor has been cloned (*13*) (Fig. 3).

The data imply that occupied CSF receptors can initiate multiple signaling cascades in the responding cell to achieve the multiplicity of known actions of the CSFs. It appears that the responding cells must themselves be able to determine which signaling cascades are generated and which types of cellular responses are elicited. This prediction that the CSFs can initiate multiple signaling cascades probably needs qualifying for mitotic signaling. In studies on the continuous murine hematopoietic cell line FDC-P1, the cells can respond to mitotic stimulation by GM-CSF, IL-3, interleukin-4 (IL-4), and interferon-γ. After complementary DNA transfection of receptor and expression of the relevant receptors, these cells can also be stimulated by M-CSF (*14*) and human GM-CSF (*15*). It is improbable that any one cell would possess six distinct mitotic signaling pathways. Thus, although the initial signals from the various bound receptors probably differ, ultimately these funnel into a common pathway reaching the appropriate chromosomal target.

Interactions Between the CSFs and Other Regulators

The four CSFs functionally interact when influencing the behavior of responding granulocytes and macrophages. These probably are direct interactions on individual cells, made possible by the co-expression of more than one type of CSF receptor, but for some responses this has not been formally proven. Although each CSF receptor contains at least one unique subunit conferring specificity, the different receptors exhibit a variety of interactions. Occupation of one type of CSF receptor by its ligand can down-modulate other CSF receptors (*16*), and binding of GM-CSF to its receptor leads to instability of the messenger RNA for the M-CSF receptor with loss of expression of the M-CSF receptor (*17*). In human cells, GM-CSF and IL-3 cross compete for receptor binding (*18*) because the receptors share a common subunit that can associate with and convert either receptor to a high-affinity form (*13, 19*). The receptor for interleukin-5 (IL-5) may also share this common subunit, and in view of the low numbers of CSF receptors on cells, this sharing of

subunits suggests the existence of receptor clustering on these cells.

Combining two CSFs leads to additive or superadditive proliferative responses (*2*). In some cases, this may be because certain precursor cells require double signaling for optimal proliferative responses. If a common final mitotic signaling pathway exists in cells, additive proliferative effects become easy to interpret. Where superadditive responses result, it may be that certain signaling cascades are rate-limited by a particular component of the cascade that can be supplemented by the second cascade. Two more hematopoietic growth factors, IL-6 and the stem cell factor (SCF) (also named mast cell growth factor, kit ligand, or Steel factor), can have direct proliferative effects in vitro at least on murine granulopoietic populations, but considerably higher concentrations are required than is the case for the CSFs (*20, 21*) (Fig. 2). Combination of SCF with IL-6 or certain CSFs can lead to enhanced proliferative responses.

If the responding progenitor cells are bipotential and can form both granulocytic and macrophage progeny, the data suggest that CSFs such as M-CSF and G-CSF can induce competitive commitment of some of the cells to produce exclusively cells of one or other lineage (*2*). This is an important principle to establish in hematopoiesis because it places regulators in a dominant role in determining the lineage of cells ultimately produced by multipotential stem cells. Work with multipotential stem cells or hematopoietic cell lines (*22, 23*) indicates that lineage-specific regulators can commit such cells irreversibly to the appropriate lineage. However, to be convincing such studies need to be extended to a clonal analysis of the initial progeny of individual multipotential cells. When mature cells are exposed to two CSFs, it might be expected that the outcome is a simple additive effect of whatever functions are usually stimulated by each CSF when acting alone. Few studies address this question, however, and some unanticipated antagonistic consequences may be uncovered. For example, stimulation with GM-CSF has been observed to suppress the G-CSF–induced rise in alkaline phosphatase in mature neutrophils (*24*).

The CSFs can also induce each other. Macrophages can be induced by IL-3 and M-CSF to produce G-CSF (*25*) and by GM-CSF to produce M-CSF (*26*). Such interactions form part of a complex network of regulatory signaling that might permit a variety of inductive cascades, some of which could be autostimulatory. The cell types most commonly implicated in such potential networks are fibroblasts, stromal cells, endothelial cells, T lymphocytes, and macrophages and involve the regulators interleukin-1 (IL-1), IL-2, IL-6, interferon-γ, and the CSFs (*27–30*). Whether such potential networks are of importance during the development of a local inflammatory lesion or in the control of basal hematopoiesis remains speculative.

The CSFs act on committed granulocyte-macrophage progenitor cells and their immediate progeny (Fig. 1). These cells cannot self-renew; the cells become expended in the consequent cell proliferation. Combination of CSFs with an agent such as SCF, which is able to stimulate progenitor cell formation from some of the more ancestral stem cells, results in a major enhancement of cell production (*21, 31*). Although single regulators can efficiently stimulate proliferation of committed progenitor cells and their progeny, the more ancestral stem cells appear to require combinations of stimuli.

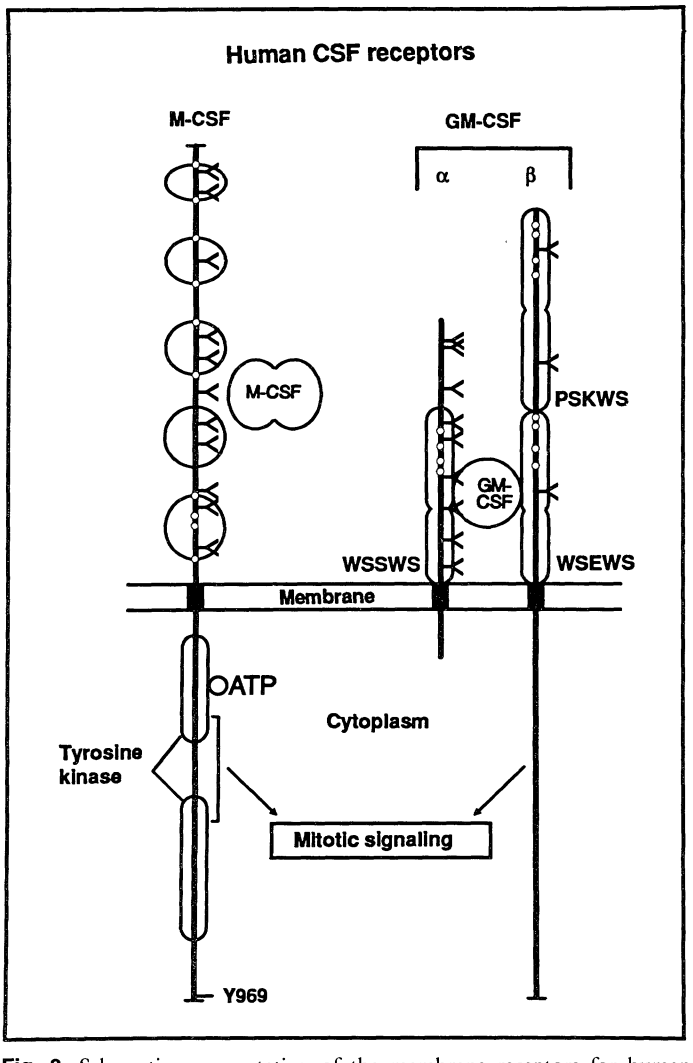

Fig. 3. Schematic representation of the membrane receptors for human M-CSF and GM-CSF. White spots on receptor chains are cysteine residues; each carbohydrate attachment site is indicated by a "Y" branching off of the main chain. The α chain alone is the low-affinity receptor for GM-CSF. A more likely representation of the actual configuration of these receptors is in (*12*). Single-letter abbreviations for the amino acid code are as follows: E, Glu; K, Lys; P, Pro; S, Ser; W, Trp; and Y, Tyr; ATP, adenosine triphosphate.

Effects of the CSFs in Vivo

Intravenous injection of recombinant CSFs into mice revealed that the CSFs have relatively short half-lives of 1 to 3 hours but more sustained concentrations can be achieved by intraperitoneal or subcutaneous injection (*2, 3*). Injection of CSF one to three times daily can produce a 10- to 100-fold rise in granulocyte-macrophage populations in the blood and peritoneal cavity that is sustained for as long as injections are continued. This increase is based on a CSF-induced increase in the number and proliferative activity of immature granulocyte-macrophage cells in the marrow and spleen.

Each CSF elicits a distinctly different pattern of response in mice, with G-CSF inducing the highest rises in peripheral blood granulocytes (*32*) and GM-CSF inducing the greatest increase in macrophages, granulocytes, and eosinophils at the intraperitoneal site of injection (*33*). Combined injection of these two CSFs retains the distinctive features of each and produces superadditive responses in some populations. Tests on the mature granulocytes and macrophages in CSF-injected animals indicate that functional activation occurs comparable with that observed in vitro (*33, 34*).

The types of cells involved in these in vivo responses parallel the range of cells responding to the CSFs in vitro. Thus, G-CSF elicits mainly granulocytic responses, with only minor rises in macrophages and no change in eosinophil populations. GM-CSF elicits rises in macrophages, eosinophils, and, at high concentrations, megakaryocytes (*33*). IL-3, the only CSF with actions in vitro on

mast cells, elicits major rises in mast cell populations, particularly in the spleen, as well as rises in granulocytic, macrophage, eosinophil, and megakaryocytic cells (*34*).

An initial expectation was that CSF-induced responses might terminate abruptly as available progenitor cells became expended. However, CSF-induced responses were sustained for as long as CSF was injected; progenitor cell numbers actually rose significantly. This indicates a substantial reserve capacity of the stem cell compartment.

There are some quantitative discrepancies between the actions of the CSFs in vivo and in vitro. G-CSF is the weakest stimulus for granulocyte proliferation in vitro but in vivo elicits the highest rises in blood granulocytes. This suggests the occurrence of significant interactions between the injected CSFs and other regulatory molecules in vivo. Combination of SCF with G-CSF in vitro produces a tenfold enhancement of resulting granulocyte formation (*21*), and a mechanism of this type could be responsible for the in vivo effects of G-CSF. This conclusion is supported by the relative inactivity of G-CSF when injected into mice with the Steel mutation, which have a defective production of SCF (*35*).

The animal studies indicated that the CSFs can be powerful regulators of granulocyte-macrophage formation and function in vivo. However, such data do not establish that the basal production of granulocytes and macrophages in the body is normally controlled by the CSFs. Because the highest concentrations of CSFs in vivo are during states of perturbation, such as infections, the CSFs might function only as emergency regulators during those states requiring rapid increases in granulocyte-macrophage production or function. Proof of the role of CSFs in controlling basal hematopoiesis requires evidence that depression of CSF concentrations leads to decreases in normal granulocyte or monocyte-macrophage levels.

For two of the CSFs, such evidence has now been produced. In dogs injected with cross-reactive human G-CSF, antibodies to human G-CSF developed that cross-inhibited canine G-CSF. These dogs developed a granulocytopenia that was reproduced in normal dogs by injection of the antibody-containing serum (*36*). In the genetic disease osteopetrosis, mice have defective macrophage formation with failure of the macrophages to form osteoclasts, a cell required for bone remodeling. Excess bone formation results that encroaches on the bone marrow and produces the abnormal state of osteopetrosis. The gene involved in this abnormality (*op*) was localized to the same region on chromosome 3 as that occupied by the M-CSF gene. Sequencing of the M-CSF gene in *op/op* mice has revealed an abnormality that prevents transcription of the 2.3-kb mRNA for M-CSF and the production of M-CSF (*37*). Thus, both G-CSF and M-CSF are required to maintain normal hematopoiesis, and it seems reasonable to predict that comparable data will eventually establish a similar status for GM-CSF and IL-3.

Documentation of the potential clinical value of the CSFs requires not only the demonstration that they can increase granulocyte and macrophage numbers but also that they can significantly enhance resistance to infections. Several experimental studies have demonstrated the ability of the CSFs to reduce mortality following infections. For example, injection of G-CSF increased by 1000-fold the ability of cytotoxic drug–treated mice to resist subsequent challenges with lethal doses of a range of microorganisms (*38*). Similarly, both GM-CSF and G-CSF have been shown to reduce mortality after otherwise lethal doses of whole-body irradiation, a situation in which many deaths are due to secondary infections (*39*).

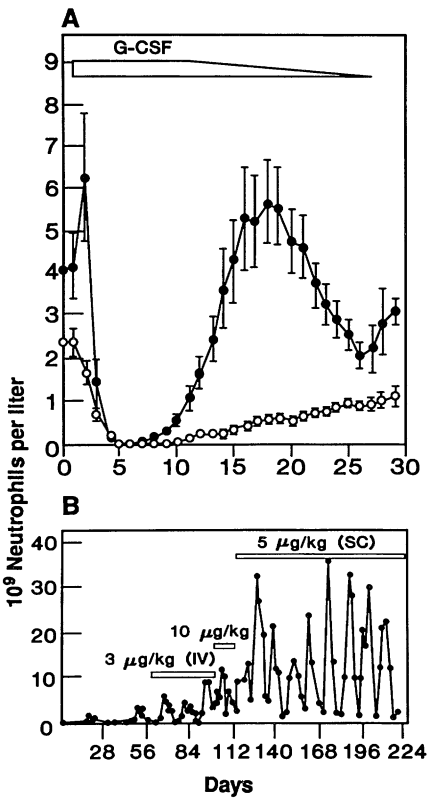

Fig. 4. Some examples of the current clinical use of G-CSF. (**A**) In individuals with lymphoma and cancer that receive autologous marrow transplants after intensive chemotherapy, injections of G-CSF (●) accelerate the recovery of adequate blood neutrophil numbers (per liter of blood), as compared to the slow recovery in historical controls (○). Data adapted with permission from Sheridan and co-workers (*50*). The wedge shape for G-CSF indicates that a decreasing concentration of G-CSF was used in the experiment. (**B**) In an individual with cyclic neutropenia, G-CSF injections do not prevent cyclical fluctuations, but they do elevate the numbers of neutrophils (per liter of blood) high enough to prevent recurrent infections. Bars indicate the time and amount of G-CSF used (in micrograms of G-CSF per kilogram of body mass). SC, G-CSF administered subcutaneously; IV, G-CSF administered intravenously. Data adapted by permission of the Massachusetts Medical Society (*N. Engl. J. Med.* **320**, 1306, 1989).

Clinical Uses of the CSFs

Clinical trials on the CSFs were commenced in 1986 and initially involved GM-CSF and G-CSF. Phase I trials established that these CSFs were able to elicit rises in blood and marrow granulocyte-macrophage populations comparable with those observed in animal studies without major toxicity (*40, 41*). Blood granulocyte amounts could be elevated in a concentration-dependent manner, and responses were maintained for as long as CSF injections were continued.

In humans, blood granulocyte numbers are normally in the range of 4000 to 6000 cells per microliter, and serious susceptibility to infections develops below 1000 cells per microliter. Conversely, during a natural response to a bacterial infection, such as pneumonia, granulocytes normally rise to 10,000 to 20,000 cells per microliter. The CSFs can raise white cell numbers beyond this, but no advantage is gained from extreme numbers; unnecessarily high numbers of monocyte-macrophages, coupled with CSF stimulation of these cells, can lead to the formation of toxic products and tissue damage (*42*).

In most clinical studies to date, the individuals studied have had subnormal hematopoiesis, either as a consequence of diseases such as acquired immunodeficiency syndrome (AIDS) (*40*), aplastic anemia (*43*), and congenital (*44*) or cyclic neutropenia (*45*) or as a consequence of cytotoxic therapy for cancer, lymphoma, or leukemia (*46–48*). The CSFs can stimulate increases in granulocyte-monocyte populations in such individuals, but responses are quantitatively restricted if the available numbers of stem and progenitor cells have been drastically depleted by disease or chemotherapy. Responses to CSF treatment are evident from the partial or complete correction of a preexisting abnormality, such as in congenital neutropenia (*44*), or by the more rapid regeneration of hematopoietic cells after cytotoxic therapy, as after bone marrow transplantation, for example (*49, 50*) (Fig. 4). CSF treatment can therefore result in a shortening of the period of intensive nursing and hospitalization.

All studies have noted some impact on the frequency of infections, although the use of CSF does not achieve absolute protection. The

most unambiguous effects have been noted in individuals with congenital or cyclic neutropenia (*44, 45*), in whom the use of CSF, for up to 3 years, has had a clear effect in reducing the occurrence of infections. As a result of these trials, two of the CSFs (G-CSF and GM-CSF) have been approved in various countries for clinical use, and clinical trials on M-CSF and IL-3 are in their early phases. Because the CSFs can functionally activate existing granulocytes and monocytes, a future extension of these clinical studies is to individuals with near-normal hematopoiesis but at risk of infections. Such individuals include those with trauma or burns or those scheduled for operations with a known risk of secondary infections.

One response noted in individuals receiving CSF treatment is a rise (up to 100-fold) in the numbers of progenitor cells in the blood (*51*). These reach concentrations comparable with those in the bone marrow, raising the possibility of using blood cells in place of or in addition to the marrow cells that are used for autologous transplantation after chemotherapy in individuals with cancer or leukemia. Initial trials of a combination of blood and marrow cells have shown an acceleration of the regeneration not only of white cells but also of platelets. This latter observation could be of major practical importance because thrombocytopenia and the consequent need for platelet transfusions remain major clinical problems in individuals receiving chemotherapy for cancer.

Sixteen hematopoietic regulators have now been produced in recombinant form and are potentially available for clinical use either alone or in various combinations. Adequate testing of these agents will be a formidable logistical problem for clinicians, the more so if the cost of introducing any one agent to the clinic remains at $50 million to $100 million. Simpler, yet still safe, testing procedures need to be devised, and more collaboration between pharmaceutical companies is required than is now the case—a challenge to such companies to modify current practices. Otherwise, clinically valuable agents may never reach the bedside.

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