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The Florey Lecture, 1991: The Colony-Stimulating Factors: Discovery to Clinical Use

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Phil. Trans. R. Soc. Lond. B 1991 **333**, 147-173
doi: 10.1098/rstb.1991.0065

The Florey Lecture, 1991. The colony-stimulating factors: discovery to clinical use

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SUMMARY

The four colony-stimulating factors, GM-CSF, G-CSF, M-CSF and Multi-CSF, are specific glycoproteins with a likely common ancestral origin which interact to regulate the production, maturation and function of granulocytes and monocyte-macrophages. Each has been purified and produced in active recombinant form. Animal studies have shown the ability of injected CSF to increase the production and functional activity of granulocytes and macrophages *in vivo* and to enhance resistance to infections. These studies have led to the current extensive clinical use of CSFs to promote the formation and function of granulocytes and macrophages in a wide variety of disease situations in which there is an associated risk of serious infections.

Although our knowledge of the control of haemopoiesis remains incomplete, the approaches used to develop the CSFs can be used to extend this knowledge, with the promise of the introduction into clinical medicine of additional effective therapeutic agents.

1. INTRODUCTION

I never met Howard Florey or heard him lecture during his occasional visits to the corridors of academic power in Australia in the 1950s and my knowledge of him remains somewhat fragmentary.

From his publications, he appears to have had an abiding interest in inflammatory responses to infections but it is not certain that he would necessarily have expressed much interest in the subject of this lecture: the colony-stimulating factors (CSFs). Logically, he should have, as the CSFs seem to be key molecules in such inflammatory responses. However, my perception of Florey is that he succeeded by single-mindedly pursuing scientific questions of direct interest to him with often little regard for the work or interests of those in other fields. I can scarcely be critical of such behaviour as I recognize this tendency in myself.

What Florey would more likely have recognized and appreciated in the CSF story is a pattern that became familiar to him during his work on penicillin. Both penicillin and the CSFs were discovered by accident, both were to require prolonged and disciplined teamwork to convert qualitative observations to purified materials and both had the obvious possibility of clinical application. For both, a difficult developmental period ensued before adequate coordination between laboratory, industry and clinic allowed the dream of clinical application to become a reality.

Whereas the intellectual intricacies of the type

Phil. Trans. R. Soc. Lond. B (1991) **333**, 147–173
Printed in Great Britain

presented by the penicillin family or the growth-factor networks seem sufficient to absorb the energies of most investigators, fortunately there have always been a few who remain unsatisfied until, by sustained intellectual and physical effort, a phenomenon is transformed to a treatment. This lecture honours such a person and those workers on the CSFs who continued the Florey tradition.

2. HISTORICAL BACKGROUND

Although many cell types in the blood and local tissues play a role in protecting the body against acute infections, two white cells – the neutrophilic granulocyte and the monocyte–macrophage – are of special importance. It was recognized early in this century that abnormally low granulocyte levels in the blood (less than 1000 per microlitre) rendered the body susceptible to infections and this basic correlation has been confirmed repeatedly (Bodey *et al.* 1966).

In health, the production of granulocytes and macrophages needs to be continuous, because both cell types are short-lived, and this production is normally sustained with impressive precision. The precursor cells generating these mature blood cells are mainly located as dispersed populations in various bones. Because of this arrangement, it has always been assumed that the most feasible system for coordinating such cell production would involve humoral agents, capable of regulating in concert these dispersed cell populations.

Attempts, using intact animals, to detect such humoral regulators of granulocyte and macrophage formation proved fruitless. It was not until the development of culture systems able to support the proliferation of murine bone marrow and spleen cells to form differentiating colonies of granulocytes and macrophages in semisolid agar medium (Bradley & Metcalf 1966; Ichikawa *et al.* 1966) that a feasible method presented itself for detecting and characterizing possible regulators of these cells.

Analysis of these cultures showed that immature granulocytic and macrophage cells could not proliferate spontaneously. Cell division needed to be stimulated by inclusion in the cultures of tissue fragments, various 'feeder' cells or medium from cultures of such cells or tissues. At that time, only one molecular regulator of haemopoietic cells was known. This was erythropoietin, which seemed sufficient, when acting alone, to control red cell formation. By analogy with this model, the initial assumption made was that a single regulator might suffice to control cell proliferation in the granulocyte–macrophage lineage. The descriptive name 'colony-stimulating factor' (CSF) was applied to the molecule postulated to be necessary to stimulate cell proliferation during granulocyte–macrophage colony formation and that might be the regulator of granulocyte and macrophage formation *in vivo*.

Efforts to determine the molecular nature of CSF and to establish that it was a genuine regulator of granulocyte and macrophage formation *in vivo* encountered unanticipated technical problems. Only minute amounts of CSF appeared to be present in, or produced by, even the richest tissue source. Not only did this necessitate an extensive search for starting materials with the highest possible apparent activity, but presented logistical problems in amassing amounts likely to be needed to permit final purification. Furthermore, as these studies progressed, it became obvious that the initial assumption, that there was only a single CSF, became progressively less sustainable. Eventually it became apparent that there were at least four distinct CSFs, quadrupling the task of characterization. When granulocyte–macrophage colonies were grown by using cells from other species, it also became evident that species-specificity existed for the CSFs, so that it became necessary to contemplate not only the purification of the four murine CSFs but also that of the corresponding four human CSFs.

The most formidable technical problem however proved to be the extensive purification required to achieve complete purification of each CSF. In the most difficult case, this required purification of one million-fold. Success in the purification of the CSFs ultimately was to depend on the development of sophisticated separative procedures such as affinity chromatography and high-performance liquid chromatography that were not in existence when the programme was commenced.

Fortunately these future problems were not foreseen when the first attempts were begun in the late 1960s to purify CSF, for it was not until 1983 that the purification of all four murine CSFs was finally accomplished (table 1).

The CSFs proved to be glycoproteins with a content of carbohydrate that varies widely according to the tissue source and the culture conditions used to produce the material. The resulting heterogeneity in size and charge often led to the appearance of multiple peaks of activity in fractions of the material being purified, necessitating revision of the sequence of purification steps with continuing uncertainty regarding the purity of the material produced. Even when purified CSF was finally produced and yielded a single amino acid sequence, it required cDNA cloning and expression of recombinant CSF to verify that the material originally purified and sequenced had indeed been CSF.

With the technology and experience now available, a project of the CSF purification type could probably be completed in six months. Even so, such projects still depend on the availability of sensitive and reproducible quantitative bioassays and starting materials with adequate levels of biological activity if they are to have a reasonable prospect of success.

CSF was detectable in human urine and this favourable combination of a cheap and abundant starting material with a convenient bioassay led to the choice of human urinary CSF as the first to be purified (Stanley *et al.* 1975). The CSF involved was of molecular mass 45 000–70 000 Da and is now known as M-CSF because of its predisposition to stimulate macrophage colony formation. The equivalent murine M-CSF was purified from medium conditioned by mouse fibroblasts in 1977 (Stanley & Heard 1977).

Meantime, a much smaller CSF of apparent molecular mass 23 000 Da was detected in medium

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Table 1. *The colony-stimulating factors*

(Estimates of the moleculr mass of the native CSFs vary according to the degree of glycosylation. At least two different forms of the M-CSF dimer are produced, both involving separate N-terminal and C-terminal processing. For further details see Nicola (1989).)

species	factor	acronym	molecular mass of core protein/Da	molecular mass of native glycosylated protein/Da
mouse	granulocyte–macrophage colony-stimulating factor	GM-CSF	14400	18000–25000
	granulocyte colony-stimulating factor	G-CSF	19100	25000
	macrophage colony-stimulating factor	M-CSF	21000 (×2)	70000–90000
		(CSF-1)	18000 (×2)	45000–50000
	multipotential colony-stimulating factor (Interleukin-3)	Multi-CSF (IL-3)	16280	18000–30000
human	granulocyte-macrophage colony-stimulating factor	GM-CSF	14700	18000–30000
	granulocyte colony-stimulating factor	G-CSF	18600	20000
	macrophage colony-stimulating factor	M-CSF	21000 (×2)	70000–90000
		(CSF-1)	18000 (×2)	45000–50000
	multipotential colony-stimulating factor (Interleukin-3)	Multi-CSF (IL-3)	15400	15400–30000

conditioned by mouse lung tissue which differed from urinary CSF in being able to stimulate both granulocytic and macrophage colony formation (Sheridan & Metcalf 1973). Purification of this molecule, now known as GM-CSF, was completed in 1977 (Burgess *et al.* 1977).

In work on CSF in the serum of mice injected with endotoxin, it was recognized that, although this serum contained high levels of M-CSF, antiserum to M-CSF did not entirely suppress colony formation. Such cultures contained low numbers of small, inconspicuous, colonies composed of mature granulocytes (Burgess & Metcalf 1980). Mouse-lung-conditioned medium also contained high levels of an apparently similar activity, and as this source material was in continuous production for work on GM-CSF purification, a second series of fractionation procedures was initiated to purify this possibly different CSF. The bioassay of this new CSF was aided by the recognition that the active factor could induce differentiation in colonies of a murine leukaemic cell line, the WEHI-3B, and a dual assay system was used to monitor purification of the molecule. This CSF, now known as G-CSF, was finally purified in 1983 as a molecule of molecular mass 25000 Da after many losses of purified material due to its extremely high hydrophobicity and stickiness to glass and plastic surfaces (Nicola *et al.* 1983).

Meantime, other experiments had documented that certain crude materials such as mitogen-stimulated lymphocyte conditioned medium or medium conditioned by WEHI-3B leukaemic cells not only could stimulate granulocyte-macrophage colony formation but also the formation of colonies of eosinophils, megakaryocytes, mast cells and mixed colonies containing cells of multiple lineages (Metcalf 1984).

Initial efforts to purify the active factors in spleen lymphocyte conditioned medium employed multiple assays for the various colony types and it slowly became evident that these multiple functions were in fact ascribable to a single factor. Meantime, a quite different line of investigation had been in progress to identify and purify a factor able to induce 20α-hydroxy steroid dehydrogenase in T-lymphocytes. Purification of this molecule (molecular mass 23000–28000 Da) under the name interleukin-3 (Ihle *et al.* 1982) resulted in the surprising observation that this factor could also stimulate the proliferation of mast cells and certain continuous haemopoietic cell lines, and was the same molecule as was eventually purified under the name of Multi-CSF (Cutler *et al.* 1985), based on its ability to stimulate the formation of colonies of multiple cell lineages.

Purification of the human analogues of GM-CSF and G-CSF was achieved, by using as starting material, medium conditioned by various human tumour cell lines found to be producing high levels of CSF (Gasson *et al.* 1984; Welte *et al.* 1985; Nomura *et al.* 1986).

The most dramatic advance in the characterization and development of the CSFs occurred with the introduction of molecular biology into this field. Within a period of three years from 1983 to 1986, complementary and genomic DNAs for each of the four murine and human CSFs were isolated either with the use of nucleotide probes based on some amino acid sequence data or by direct expression screening (table 2). This allowed the full amino acid sequence of each CSF to be determined. Much more important was the development of expression systems capable of producing relatively large amounts of recombinant CSF and the demonstration that these recombinant CSFs exhibited the same range of actions as the corresponding native CSFs with equivalent biological activity per milligram protein.

These developments solved the crippling logistical problems for those groups capable of purifying CSFs

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Table 2. *The genes encoding the colony-stimulating factors*

(For detailed referencing of the original publications, see Nicola (1989).)

species	factor	chromosomal location	number of exons	mature cytoplasmic mRNA/kb
mouse	GM-CSF	11	4	0.78
	GM-CSF	11	5	1.6
	M-CSF	3	—	4.5, 3.8, 2.3, 1.4
	Multi-CSF (IL-3)	11	5	1.0
human	GM-CSF	5q21–q31	4	0.78
	G-CSF	17q21–q22	5	1.7
	M-CSF	5q33.1	10	4.5, 3.3, 2.6, 1.6
	Multi-CSF	5q21–q31	5	1.0

for detailed study, and when the mass production of recombinant CSFs was taken over by various gene cloning and pharmaceutical companies, purified recombinant CSFs became available for most experimental haematology groups and, quite rapidly, for clinical trials. The first clinical trials on GM-CSF and G-CSF were in fact commenced in 1986, which represented a remarkably rapid transition through the phases of testing in experimental animals and preclinical primate trials.

3. THE GENERAL PATTERN OF HAEMOPOIETIC REGULATION

Some general points need to be made at this stage to place the CSFs in the general framework of information on the molecular control of haemopoietic populations.

The eight major lineages of haemopoietic cells are generated by the proliferative activity of a small and heterogeneous population of multipotential haemopoietic stem cells with a capacity for self-generation. Stem cells also have the ability to generate relatively large numbers of progeny known as committed progenitor cells (Metcalf 1984). These latter are the cells forming haemopoietic colonies in semisolid cultures. Progenitor cells have no capacity for self-generation and are usually restricted in differentiation potential to form cells of a single lineage or at most a limited number of lineages. Thus granulocyte–macrophage-committed progenitor cells can form granulocyte, macrophage or both granulocyte and macrophage progeny. The proliferative potential of committed progenitor cells can be large and result in the generation of up to 10^5 maturing progeny, the progeny passing through the familiar morphological changes culminating in the formation of the mature cells released *in vivo* into the peripheral blood.

Complicating this hierarchical arrangement of cell formation is the extreme heterogeneity of cells in proliferative and lineage potential at all ancestral stages. Although techniques now exist for obtaining pure populations of stem and progenitor cells, such cells are so heterogeneous that many apparently simple experiments to analyse their control by regulatory molecules can be extremely difficult to interpret.

The final complication in haemopoiesis is the fact that mature cells in some lineages remain capable of extended cell division. Mature cells in these lineages can therefore come from two sources: the *de novo* formation of cells by precursors in the marrow or the proliferation of mature cells in peripheral tissues. Cells having such a potential for a double origin are mast cells, eosinophils, macrophages and both T- and B-lymphocytes.

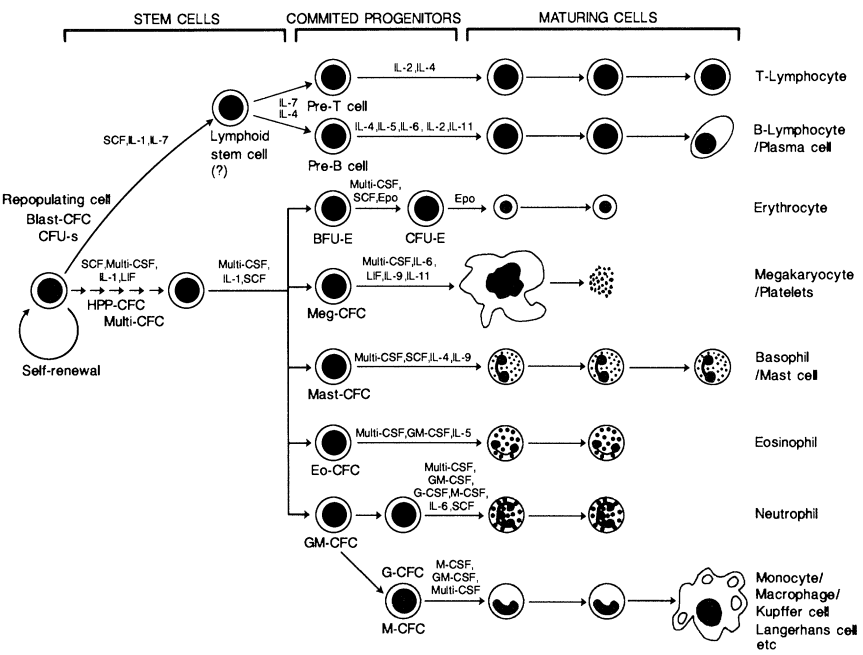

Figure 1. A schematic representation of haemopoiesis indicating that self-generating stem cells form committed progenitor cells, each with a capacity to generate large numbers of maturing cells. Multiple regulatory molecules act to control the formation of cells within each lineage. These regulators are shown in more detail in table 3.

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Table 3. *Regulatory molecules controlling haemopoiesis in the mouse*

(E = erythroid, M = macrophage, G = neutrophilic granulocyte, Eo = eosinophil, Meg = megakaryocytic, Mast = mast cell, Multi = multipotential, Stem = stem cell, T = T-lymphocyte, B = B-lymphocyte. Molecular mass of non-glycosylated polypeptide. M-CSF and IL-5 are dimers.)

regulator	acronym	molecular mass of Polypeptide/ Da	responding cells
erythropoietin	Epo	18400	E, Meg
granulocyte–macrophage colony-stimulating factor	GM-CSF	14400	M, G, Eo, Meg, E, Multi
granulocyte colony-stimulating factor	G-CSF	19100	G, M
macrophage colony-stimulating factor	M-CSF	21000(×2) 18000(×2)	M, G
multipotential colony-stimulating factor	Multi-CSF(IL-3)	16200	M, G, Eo, Meg, Mast, E, Multi, Stem
interleukin-1	IL-1	17900	T, stem
interleukin-2	IL-2	19400	T, B
interleukin-4	IL-4	14000	T, B, Mast, G, M
interleukin-5	IL-5	13300(×2)	Eo, B
interleukin-6	IL-6	21700	B, G, Meg, Stem
interleukin-7	IL-7	14900	B, T
interleukin-9	IL-9	14200	T, Mast, E
interleukin-10	IL-10	18700	Mast, B
interleukin-11	IL-11	21000	M, Multi, B, Meg
stem-cell factor	SCF	18400	Stem, Multi, G, Mast
leukaemia inhibitory factor	LIF	20000	Meg

The control mechanisms regulating these cellular events are of two general types: (i) stromal cell control, involving the influence of specialized cells in the microenvironment of haemopoietic cells and acting either by cell-contact mechanisms or by the production of short-range humoral factors, and (ii) molecular regulators, which may be produced and act locally or be products of distant tissues and then exhibit the biological behaviour of classical hormones (Metcalf 1984, 1988).

It is now recognized that stromal cells have the capacity to produce a number of the known molecules regulating haemopoiesis, but it remains unclear whether they might exhibit, in addition, unique functional activities able to enhance the proliferation of haemopoietic cells and particularly of stem cells.

Sixteen distinct haemopoietic regulatory molecules have so far been identified with proliferative actions on cells of one or other haemopoietic lineage (figure 1, table 3). Cells of each lineage are able to be controlled by more than one regulator and often a particular regulator can act on cells of several lineages for which there is little evidence of immediate ancestral relationship. For example, interleukin-6 has actions on the unlikely combination of granulocytes, megakaryocytes and B-lymphocytes.

The overall pattern of these regulators is therefore one of redundancy, with overlapping actions that can involve cells of different lineages or hierarchical subsets. In addition, a network of secondary regulatory factors exists which can influence the production of the primary haemopoietic growth factors, but with no direct action on haemopoietic cells. The colony-stimulating factors are typical examples of haemopoietic regulators, and the principles emerging from the more complete information on the CSFs are likely to prove valid for the other regulators acting on haemopoietic populations.

4. THE CSFs AND THEIR RECEPTORS

CSFs are glycoproteins with a variable content of carbohydrate, either N-linked or O-linked (Nicola 1989). The carbohydrate moiety of the CSFs is not involved in receptor binding and is not necessary for biological action of the CSFs either *in vitro* or *in vivo*. The most likely role of the carbohydrate is to influence the distribution or half-life of the CSFs *in vivo*, although comparison of non-glycosylated with glycosylated material has so far not shown dramatic differences.

The polypeptide component of three of the CSFs is in the form of a single chain, while the fourth (M-CSF) is a dimer of two apparently identical monomeric units. The size of these chains ranges from 14000 to 21000 Da (table 1). Paired cysteine residues in the polypeptide chains are linked by disulphide bridges that are necessary to hold the molecules in a biologically-active configuration (figure 2). Similar disulphide bridges link the monomeric units of M-CSF to form a functionally active dimer and the monomeric unit itself is biologically inactive.

Multiple regions of the CSF polypeptides are required for biological activity and presumably combine to form the receptor-binding domain. Because small fragments of the CSF polypeptides lack biological activity and only low levels of native CSFs are present in tissues, the only practicable method for mass-

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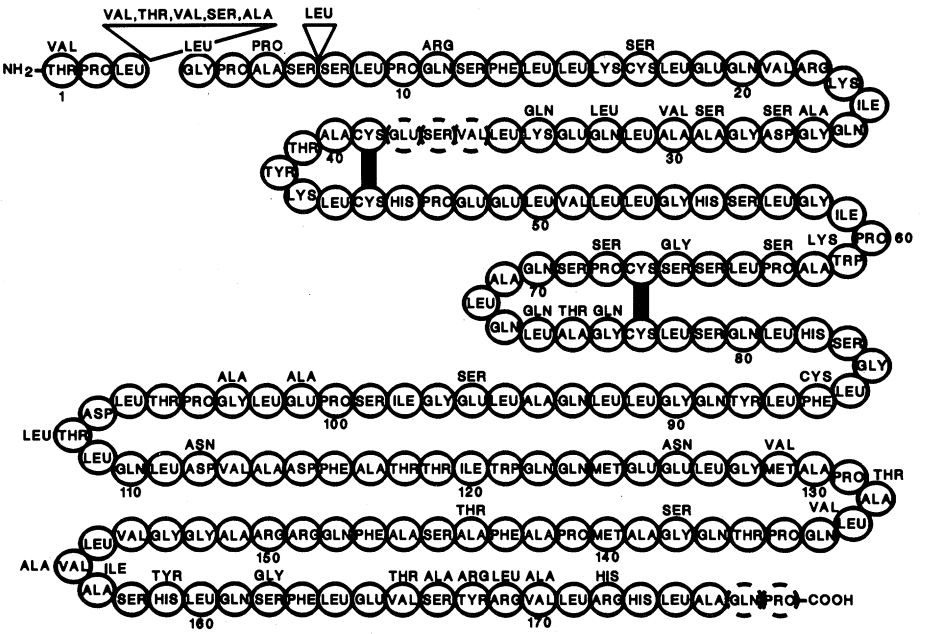

Figure 2. Schematic representation of the polypeptide chain of human and murine G-CSF. The primary sequence shown and numbering are for human G-CSF. Insertions in the mouse sequence are shown by triangles and deletions by dashed circles. Substitutions in the mouse sequence are shown above the human sequence. Cysteine–cysteine disulphide bonds are shown by heavy bars. Reproduced with permission from Nicola (1990).

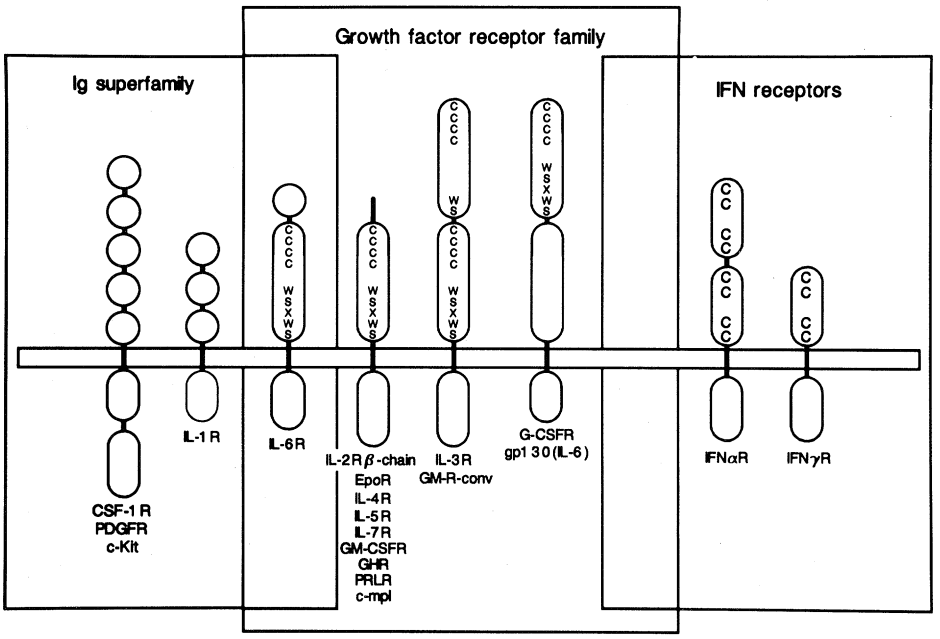

Figure 3. Schematic representation of the structure of the CSF membrane receptors. The receptor for M-CSF has a tyrosine kinase domain and shares homology with the platelet-derived growth-factor receptor. The remaining CSF receptors show homology in their extracellular domains with the receptors for erythropoietin, prolactin, growth factor and a number of interleukins. The regulator receptor family has some similarities to both immunoglobulins and the interferon receptors.

producing CSFs is the production of recombinant CSF using bacterial, yeast or mammalian expression systems. The complete murine Multi-CSF polypeptide has been chemically synthesized (Clark-Lewis *et al.* 1986) but had a much lower biological activity per milligram protein than the native or recombinant molecules, presumably because of cumulative errors occurring during the sequential addition of amino acids in the synthetic process.

Data from amino acid sequencing of intact CSFs or peptide fragments, extended by deduction of the full amino acid sequences from cloned cDNAs for the CSFs, have shown no significant sequence homology between the CSFs or with any other growth factors or polypeptides. Furthermore, there is no similarity in predicted secondary structure between the four CSFs. The cDNAs for each CSF encode a typical signal region, permitting secretion after cleavage.

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It has been shown, at least for GM-CSF, that the 3′ untranslated region of the mRNA contains an A–U-rich region able to modify the stability of the mRNA (Shaw & Kamen 1986). In some situations leading to increased cellular CSF mRNA levels and CSF production, increased rates of transcription of mRNA are not involved but rather the induction of increased stability in existing mRNA.

M-CSF is the only CSF for which convincing evidence exists for the formation of alternate transcripts that can generate protein products able to be displayed on the cell membrane (Rettenmier *et al.* 1987). Cells manipulated to selectively display membrane-bound M-CSF have been found capable of stimulating macrophage precursors after effective cell contact is made (Stein *et al.* 1990).

The level of amino acid homology between murine and human equivalent CSF polypeptides varies from 82% for M-CSF, 78% for G-CSF and 52% for GM-CSF to only 28% for Multi-CSF. Paralleling these differences, human G-CSF and M-CSF are also active on murine cells, whereas human GM-CSF or Multi-CSF are not.

Although some attempts have been made to determine the likely three-dimensional structure of the CSFs by using data from the biological activity of CSF fragments, the products of mutein cDNAs and mapping using monoclonal antibodies (Kaushansky *et al.* 1990), definitive data on these structures awaits crystallographic and NMR studies now in progress.

The dissimilarity of the CSF polypeptides appeared to undermine the original concept that these were likely to be a related family of regulators because of their similar biological actions, often on the same target cells. However, newer information on the chromosomal location and regulation of the CSF genes, and particularly on the structure of the CSF receptors, does support the original concept that these are a family of regulators, derived by evolution from a common ancestral precursor.

Each CSF is encoded by a single unique gene. Arguments have been raised, based on the general similarity of structure of these genes that they may be related ancestrally (Clark & Kamen 1987). More convincing for this view is the chromosomal clustering of haemopoietic growth-factor genes to be found both in the mouse and man (table 2). The genes for GM-CSF and Multi-CSF exhibit a particularly close proximity on chromosome 11 in the mouse and chromosome 5 in man, with some evidence for common or shared transcriptional regulation regions (Gough & Nicola 1990). In both species, these genes also lie in proximity to the genes for two other haemopoietic regulators, IL-4 and IL-5 (Van Leeuwen *et al.* 1989). In man, somewhat more removed on chromosome 5, are the genes for M-CSF and its receptor, *c-fms*, but this relationship breaks down in the mouse where the gene for M-CSF is on chromosome 3 and for *c-fms* on chromosome 18.

The first CSF receptor to be identified was that for M-CSF, which was recognized to be the product of the protooncogene *c-fms* (Sherr *et al.* 1985). The M-CSF receptor is a typical single-chain transmembrane glycoprotein, the cytoplasmic portion of which contains a tyrosine kinase domain likely to be involved in ligand-initiated signalling. Subsequently, cDNAs have been cloned for the receptors for GM-CSF, Multi-CSF and G-CSF (Gearing *et al.* 1989; Itoh *et al.* 1990; Fukunaga *et al.* 1990). Although each is also a single-chain transmembrane glycoprotein, they differ from the M-CSF receptor in not containing a tyrosine kinase domain. This requires that ligand-initiated signalling be initiated by a different mechanism, most likely involving an associated membrane protein as is the case for the prototype IL-2 and IL-6 receptors. To date, only one of these likely accessory molecules for CSF receptors has been cloned – that for the human GM-CSF receptor (Hayashida *et al.* 1990), which converts the low-affinity receptor to one of high affinity.

The striking feature of these latter CSF receptors is the homology they exhibit in their extracellular domains (figure 3). This involves a tryptophan–serine–X–tryptophan–serine motif and sets of four conserved cysteine residues. These show a clear relationship, not only between the three CSF receptors, but also with the receptors for erythropoietin, interleukin-2β-chain, growth hormone, prolactin, IL-4, IL-6 and IL-7. Thus these receptors now form a superfamily of growth factor receptors with links on the one hand to the immunoglobulin constant domains and on the other to the interferon receptors (Bazan 1990).

Not only have the CSF receptors retained evidence of their common ancestral origin more clearly than the CSFs themselves, but the GM-CSF and Multi-CSF receptors may have remained linked functionally in that they may share a common accessory molecule. This may be the reason why these two receptors on human cells exhibit an apparent capacity to cross-bind the inappropriate CSF (Park *et al.* 1989). With murine and, to a degree, human cells it has also been shown that cross-down-modulation of CSF receptors can occur following binding of one type of CSF to its receptor (Walker *et al.* 1984). This occurs in a fixed hierarchical sequence but the mechanisms involved and the biological consequences for the affected cells remain conjectural (Nicola 1989).

The CSFs are all highly active molecules able to elicit proliferative responses in 50% of the responding cells at a concentration of approximately 200–500 pg ml^{-1} (figure 4). Two classes of CSF receptors have been noted – high-affinity and low-affinity, with the differing affinities likely to be based mainly on differing off-rate kinetics. While high-affinity receptors normally would be expected to mediate the biological effects of a CSF, evidence has been obtained that low-affinity GM-CSF receptors can elicit proliferative responses (Metcalf *et al.* 1990). In general, the binding of CSF to its receptor is extremely rapid, as is internalization of the bound complex. Thereafter, different cell types can handle the same CSF–receptor complex in a quite different manner (Nicola 1989), although in most cells the half-life of the bound receptor complex is relatively long (10–20 h).

Granulocyte–macrophage cells can co-express receptors for different CSF, and indeed most of the cells, regardless of maturation stage, co-express receptors for

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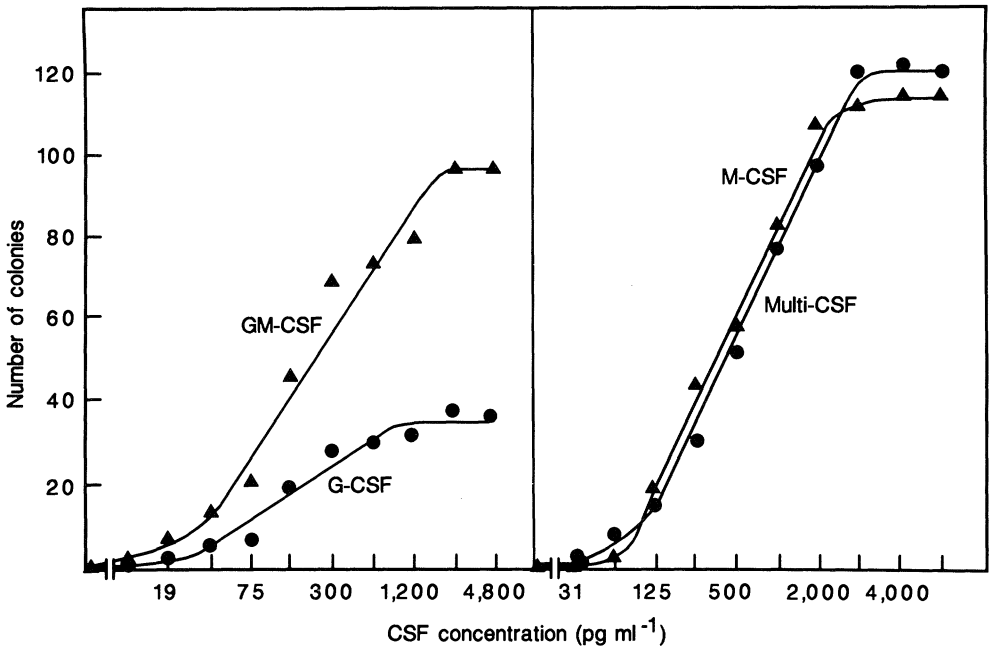

Figure 4. Stimulation of granulocyte–macrophage colony formation in cultures of 75 000 C57BL mouse bone-marrow cells by purified recombinant colony-stimulating factors. Note that although all four CSFs have a similar specific activity, G-CSF stimulates the formation of fewer colonies than do the other CSFs.

all four CSFs. This arrangement permits two or more CSFs to interact with the same cell and modulate the resulting response. The number of CSF receptors on individual cells is relatively small (up to a few hundred, on most cell types). Despite the paucity of CSF receptors, biological responses are achievable when CSF binds to only a small fraction of available receptors (Nicola 1989). The ability of bound CSF receptors to induced transmodulation of other CSF receptors, even when total receptor numbers are so small, suggests either some spatial clustering of receptors or the existence of some submembrane structure permitting interactions at a considerable distance.

5. THE BIOLOGICAL ACTIONS *IN VITRO* OF THE CSFs

The CSFs were purified on the basis of their mandatory role in stimulating the proliferation *in vitro* of granulocyte–macrophage precursors to form colonies of differentiating granulocytes and macrophages. However, the CSFs exhibit widely differing additional actions on granulocyte–macrophage populations. Indeed the CSFs were the first agents for which it became obvious that proliferative factors can also influence the maturation and functional activity of responding cells.

The evidence supporting each of the functions of the CSFs has been discussed elsewhere (Metcalf 1984, 1987), and here the older information will merely be used to set the context for additional newer information.

(*a*) *Stimulation of cell proliferation*

The four CSFs exhibit distinctive differences in their proliferative action in clonal cultures of murine marrow cells stimulated by intermediate concentrations of CSF. Both GM-CSF and Multi-CSF stimulate the formation of approximately equal numbers of granulocytic, granulocyte–macrophage and macrophage colonies together with the formation of some eosinophil colonies. Multi-CSF differs from GM-CSF in also stimulating the formation of megakaryocyte and mast-cell colonies. G-CSF characteristically stimulates the formation of relatively low numbers of small colonies, most of which contain mature granulocytes, and M-CSF preferentially stimulates macrophage colony formation although smaller numbers of granulocyte and granulocyte–macrophage colonies also develop.

The dose–response curves for stimulation of colony formation by the four CSFs are remarkably similar in cultures of murine marrow cells, despite the somewhat differing total number of progenitor cells responding to the different CSFs (figure 4). Estimates of specific activity vary, particularly for recombinant CSFs, where there can be some variation in the efficiency of refolding of the polypeptide chain, but are usually in the range 1×10^8 to 3×10^8 U mg^{-1} (50 Units/ml^{-1} is defined as the concentration of CSF stimulating the formation of 50% of maximal colony numbers). As colony numbers increase, so does average colony size, owing to the progressively greater number of progeny generated by individual progenitor cells as the concentration of CSF rises.

Comparative studies on the behaviour of paired daughter cells of individual progenitor cells documented that the CSF concentration has a direct influence on the length of the cell cycle of proliferating cells and on the total number of progeny produced (Metcalf 1980, 1985).

Interrupted exposure of progenitor cells to CSF stimulation can elicit some proliferation, suggesting that CSF-initiated signalling might be required only

during a limited phase of the cell cycle. However, such proliferation is submaximal for the CSF concentration used and the long intracellular half-life of bound CSF-receptor complexes suggests that it is more likely that CSF-initiated signalling is required throughout the cell cycle.

An interesting aspect of CSF-stimulated cell proliferation, seen most clearly with GM-CSF acting on murine cells, is the increase in range of cell lineages able to respond as CSF concentrations are increased. At very low concentrations of GM-CSF only macrophage proliferation is elicited. With intermediate concentrations both granulocyte and macrophage proliferation are stimulated, and at slightly higher concentrations, GM-CSF becomes an effective stimulus for eosinophil proliferation. With concentrations above these, GM-CSF exhibits a capacity to stimulate the proliferation of megakaryocyte precursors and some multipotential and erythroid precursors (Metcalf *et al.* 1986*b*).

The number of receptors for GM-CSF on cells in these various lineages does not exhibit a corresponding range of variation and receptors appear to be identical in nature on cells in the different lineages (Nicola 1989). This suggests that the differing differentiation or maturation programmes operating in cells of the different lineages must be able to influence the quantitative responsiveness of the cells. An alternative possible interpretation is that qualitatively different mitotic signalling cascades might be elicited in cells of different lineages. The likely existence of accessory molecules either in the membrane or cytoplasm that enhance or mediate receptor signalling has been noted above and these might conceivably differ in cells of different lineages. In support of this possibility, observations on the manner in which cells bind and internalize CSFs have provided clear evidence that (i) the same cell type handles different CSFs differently and (ii) that different cells can handle the same CSF differently (Nicola *et al.* 1988).

The notion that multiple types of signalling cascades may be able to elicit a mitotic response fits well enough the fact that multiple regulators are able to elicit the proliferation of immature cells in a particular lineage. For example, as is shown in figure 1, G-CSF, GM-CSF, Multi-CSF, M-CSF, IL-6 and SCF can all stimulate the proliferation of granulocytic precursors while GM-CSF, Multi-CSF and IL-5 can stimulate the proliferation of eosinophil precursors. The receptors for these various factors are unique and it is reasonable to anticipate that there would be some differences, at least in the initial signalling events following receptor binding.

This highly complex picture in which multiple, mitotic signalling cascades may be required to mediate the action of different regulators on a cell makes the assumption that the same precursor cell can indeed respond to as many as six regulators. This proposition is supported by the presence of receptors for all four CSFs on most immature and mature granulocytic and macrophage cells. By implication, the same situation exists for most individual progenitor cells but this has not been experimentally verified because individual precursor cells can only be tested once to establish the presence or absence of receptors for a particular CSF.

The general question of how many unique mitotic signalling pathways an individual cell might possess has been explored experimentally by using cloned continuous haemopoietic cell lines that remain factor-dependent. The FDC-P1 cell, being responsive to proliferative stimulation by GM-CSF or Multi-CSF, has been useful in this context. Retroviral insertion of the M-CSF receptor into such cells now allows them to be stimulated to proliferate by M-CSF (Rohrschneider & Metcalf 1989). Similar experiments have been done using the erythropoietin receptor and the human GM-CSF receptor. Again, such cells can now respond to stimulation by the appropriate regulators. Furthermore, FDC-P1 cells can also exhibit a limited proliferation in response to stimulation by IL-4 or γ-interferon. Thus, FDC-P1 cells are able to respond by proliferation when stimulated by at least seven different regulators, involving seven different receptors. One of these receptors was from a foreign species and it would be unreasonable to suppose that special signalling pathways pre-existed in FDC-P1 cells awaiting the insertion of this foreign receptor. The likelihood that any one cell might possess seven unique mitotic signalling pathways is quite improbable. It is more likely that only a single final pathway for mitotic signalling exists and that where differing signals originate from differing receptors, such signals ultimately enter a common signalling cascade.

This discussion of mitotic signalling cascades is not merely an exercise in theoretical model building, because the CSFs and other regulators display marked additive or superadditive proliferative actions when combined either *in vitro* or *in vivo* and these require explanation.

For example, if M-CSF and G-CSF are used as a combined stimulus for colony formation there is an increase in the number of mixed granulocyte–macrophage colonies developing, presumably due to competitive differentiation commitment (see below). There is also an additive effect on total colony numbers and an overall increase in the size of all colonies (Metcalf 1984). This is surprising because G-CSF is a characteristic stimulus for small granulocyte colony formation while M-CSF is a relatively selective stimulus for macrophage colony formation.

Although use of a common mitotic signalling cascade can explain an additive effect of two regulators, a superadditive effect suggests a more complex situation where some cascade component may be limiting, for example in the G-CSF-initiated response, but is able to be provided by the M-CSF-initiated response.

(*b*) *Differentiation commitment*

Evidence from an analysis of the behaviour of paired daughter cells of granulocyte–macrophage progenitor cells showed that the concentration and type of CSF used to stimulate the first two to three divisions can have an irreversible effect on the differentiation lineage of the progeny produced by the precursor cells. For example, stimulation of the first divisions by M-CSF

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can irreversibly commit the cells to the formation of macrophage progeny, regardless of what CSF is used to stimulate subsequent cell proliferation (Metcalf & Burgess 1982).

This type of commitment action was unusual at the time in implying that an extrinsic protein regulator could induce an irreversible change in gene programming in a responding cell. More recent work on nuclear transcription factors is identifying the possible mechanisms by which inductive events of this type can be achieved by regulator-initiated signalling, although it is still unclear how such changes are rendered irreversible.

The principle at issue is of particular importance in understanding fundamental events in haemopoiesis. If firmly established, it places in the hands of the regulator molecules an immensely powerful mechanism for influencing the type of progeny produced. Thus, if multipotential stem cells express receptors for a multiplicity of regulators, the actual type of progeny produced could be determined by the first regulator effectively interacting with the cell for a sufficiently long period. Under conditions where two or more regulators are used simultaneously, competition for commitment would be expected. Competition of this type has been documented for the simpler situation with bipotential granulocyte–macrophage progenitor cells able to respond to combinations of CSFs (Metcalf *et al.* 1982).

Similarly there are now multipotential haemopoietic cell lines available for analysis of their response to CSF combinations where the data again suggest that agents like G-CSF or M-CSF can induce corresponding granulocyte or macrophage commitment (Whetton & Dexter 1989; Heyworth *et al.* 1990).

Commitment of another type has been observed when pre-B-lymphocyte cells were induced to express the M-CSF receptor by insertion of *c-fms* mRNA. Culture of such cells in the presence of M-CSF led to the emergence of cells with the stable phenotype of macrophages (Borzillo *et al.* 1990).

(*c*) *Induction of maturation*

Stem and progenitor cells have the morphology of undifferentiated mononuclear cells. The progressive morphological changes exhibited by the progeny of these cells, (for example, the sequential formation of myeloblasts, promyelocytes, myelocytes, metamyelocytes then neutrophils) is the process termed 'maturation'. Maturation events are made possible by the preceding process of differentiation commitment, but commitment itself is not immediately evident by any change in cellular morphology. Maturation in any one lineage must require a series of tightly coordinated genetic inductive events of great complexity to achieve with fidelity the major changes in cell morphology and function.

The complexity of the events required during maturation makes it improbable that a single regulator, even if able to initiate multiple signalling cascades, could hope to influence in detail such a multiplicity of events. It might, however, be reasonable to expect that a regulator such as a CSF might be able to initiate maturation and set in train a self-perpetuating cascade of integrated inductive events.

Proof that the CSFs can initiate maturation was difficult to obtain because the CSFs are required to maintain cell viability *in vitro* (see below) and the behaviour of healthy control cells could not be monitored *in vitro* in the absence of CSF. This difficulty has now been overcome by the use of continuous haemopoietic cell lines in which the use of relatively low concentrations of Multi-CSF can maintain cell viability and basal proliferative activity without initiating maturation. Addition to such cells of G-CSF was then shown to be able to actively initiate maturation with the ultimate formation of mature post-mitotic progeny (Valtieri *et al.* 1987; Heyworth *et al.* 1990).

In view of the apparently irreversible nature of differentiation commitment, it was unexpected to observe that maturation changes can be reversible. The system used was the FDC-P1 cell line into which the M-CSF receptor was inserted using a retroviral vector. When such cells were then stimulated by M-CSF, they underwent striking morphological changes with the formation of primary granules. If the cells were then transferred to Multi-CSF, these changes proved to be reversible (Rohrschneider & Metcalf 1989). Although this system is quite artificial, the results indicate the need for some caution in assuming that commitment and maturation need necessarily be irreversible events.

(*d*) *Differentiation commitment in myeloid leukaemic cells*

Myeloid leukaemia is a clonal neoplasm often associated with defects in maturation of the leukaemic cells. The progressive expansion of the leukaemic population is essentially due to the capacity of the stem cells in the leukaemic clone to exhibit an excessive level of self-generation (Metcalf 1989*a*).

It has been observed with several well-characterized myeloid leukaemic cell lines that the CSFs can have obvious actions on such cells, most dramatically seen as the induction of maturation. As noted earlier, G-CSF-induced differentiation in the murine WEHI-3B cell line was one of the bioassays used to monitor the original purification of this regulator (Nicola *et al.* 1983). With other leukaemic cell lines such as the human lines HL60 or U937, little obvious change occurs after culture with G-CSF or GM-CSF other than the expression of certain membrane markers normally appearing during maturation (Begley *et al.* 1987; Maekawa & Metcalf 1989).

What both types of leukaemic cell lines display in common in responding to CSF is a reduction in the level of stem-cell self-generation, the key property responsible for maintenance and expansion of the leukaemic clone. Thus reduced stem-cell self-generation can occur regardless of whether or not the cells also exhibit maturation. Continued culture of suitable leukaemic cells in the presence of CSF can lead to complete suppression of the leukaemic population. The

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action of CSFs on myeloid leukaemic cells has the same general nature as their differentiation commitment action on normal progenitor cells (Metcalf 1989*a*) and may even involve common nuclear transcription events.

This action of the CSFs has important potential applications in the management of myeloid leukaemia in man. At least G-CSF, GM-CSF and M-CSF can exhibit this type of action, the most effective molecule varying with the particular leukaemic population under study (Miyauchi *et al.* 1988; Maekawa & Metcalf 1989). As with other aspects of CSF action, combinations of CSFs can be more effective than single agents. Certain other haemopoietic regulators such as IL-6 and LIF can have similar actions on leukaemic cells (Metcalf 1989*b*; Lotem *et al.* 1989) and again combinations of CSFs with these regulators leads to an enhanced suppression (Maekawa *et al.* 1989).

Many leukaemic cell lines appear refractory to this action of the CSFs, either because of failure to express the appropriate receptor on their membranes, or because of a profound derangement of gene programming preventing a suitable response.

(*e*) *Functional stimulation*

The CSFs have a number of direct actions on granulocytic and macrophage cells unrelated either to proliferation, differentiation commitment or maturation. The most basic of these is the ability of the CSFs to maintain an intact membrane transport system, variously measurable by intracellular ATP levels (Whetton & Dexter 1983), glucose transport (Hamilton *et al.* 1988) or sodium/potassium fluxes (Vairo & Hamilton 1988). As a consequence, granulocyte–macrophage precursor cells cannot survive *in vitro* in the absence of an adequate concentration of one or other of the CSFs (Metcalf & Merchav 1982; Tushinski *et al.* 1982; Metcalf 1985), and even the life-span of mature cells *in vitro* is influenced by CSFs (Begley *et al.* 1986; Lopez *et al.* 1986). It seems unlikely that these actions of the CSFs require signalling to the nucleus since this type of membrane function is most likely to be controlled by events occurring in the submembrane cytoplasm.

Slightly more complex are the demonstrable actions of CSFs in enhancing a range of functional activities of mature granulocytes and macrophages which includes adherence (Arnaout *et al.* 1986; Deveaux *et al.* 1989), chemotaxis (Gasson *et al.* 1984; Wang *et al.* 1987), phagotycosis (Fleischmann *et al.* 1986; Williamson & Brown 1987), increased superoxide production (Vadas *et al.* 1983; Wing *et al.* 1985; Weisbart *et al.* 1987), cytotoxicity for cells or microorganisms (Handman & Burgess 1979; Wing *et al.* 1982; Lopez *et al.* 1983; Ralph *et al.* 1983; Villalta & Kieszenbaum 1986; Grabstein *et al.* 1987; Karabassi *et al.* 1987; Lee & Warren 1987; Ralph & Nakoinz 1987) and the increased synthesis of a variety of products of these cells such as prostaglandin E (Kurland *et al.* 1979), interferon (Warren & Ralph 1986), tumour necrosis factor (Cannistra *et al.* 1987; Warren & Ralph 1986), interleukin-1 (Moore *et al.* 1980; Fleit & Rabinovitch 1981), plasminogen activator (Lin & Gordon 1979; Hamilton *et al.* 1980; Lyberg *et al.* 1987) and other growth factors and cytokines (Motoyoshi *et al.* 1982; Metcalf & Nicola 1985; Horiguchi *et al.* 1987).

These effects are relatively rapid in onset and require the continuing presence of CSF at concentrations similar to those eliciting proliferative responses. In some cases at least, the responses require increased transcription of relevant mRNAs whereas in other instances the effects may merely involve cytoplasmic events. Depending on circumstances, such as the proximity to other relevant cells or simply cell crowding, some of these responses are potentially autocatalytic following the activation of regulator networks.

In an actual *in vivo* situation involving local tissue damage or a local infection, these CSF-induced responses ensure the accumulation of mature granulocytes and macrophages at the site and then their functional activation. Because of the ability of the CSFs to be produced locally (see below), significant responses of this type need not involve systemic rises in CSF levels or the increased production of new cells.

Surprisingly little information is available on the consequences of CSF combinations on functional activation and this is a subject in need of further investigation. There is a possibility that a response induced by one CSF may be negated by a response to another CSF. Furthermore, not all the consequences of this functional stimulation are necessarily beneficial for the whole organism. Apart from elevating the production of molecules with potential toxicity for tissue cells, which will be discussed later, it may be that even antimicrobial actions are not always enhanced by CSF action. For facultative intracellular parasites, enhanced phagocytosis by macrophages may simply accentuate an infection if not accompanied by a corresponding elevation of intracellular killing.

A possibly similar complexity has been documented with HIV infection of monocytes, where stimulation by GM-CSF or M-CSF has been reported to enhance retroviral production in the stimulated monocytes (Folks *et al.* 1987; Koyanagi *et al.* 1988). Such activation has not been observed in response to stimulation by G-CSF (Koyanagai *et al.* 1988) and is not observed with GM-CSF in the presence of the agent, zidovudine (Hammer *et al.* 1990).

(*f*) *Comment*

The observed actions of the CSFs *in vitro* indicate that they are versatile molecules, able to control not only the formation and maturation of granulocytes and macrophages but also to regulate significant aspects of the functional activity of the fully mature cells. This is a complex array of actions and it is proper to consider how critical the role of the CSFs is in these various events.

With respect to their proliferative effects, the CSFs appear to be mandatory regulators for granulocyte and macrophage production. Both IL-6 and SCF have some capacity to stimulate granulocytic formation but the concentration of these latter regulators required is 10–1000-fold higher than for the CSFs. Accepting the

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dominant role of the CSFs, it must be recognized that the committed granulocyte–macrophage progenitors are a transit, non-self-renewing population. The ability of the CSFs to ensure continued granulocyte–macrophage production therefore depends on a continuing supply of new progenitor cells generated by more ancestral stem cells. Any agent active in stimulating progenitor cell formation by stem cells must also have an influence on the production of granulocytes and macrophages. Although both Multi-CSF and GM-CSF have some ability to stimulate progenitor cell formation, at least three other agents – SCF, IL-1 and IL-6 – have an influence on this process.

Although the CSFs certainly can influence the functional activity of mature cells, they are unlikely to play the dominant role they play in controlling cell production. For example, agents such as γ-interferon, IL-1 and endotoxin have strong actions on macrophage function and the CSFs might simply be additional agents with somewhat similar actions.

The evidence from the analysis of mitotic signalling suggests strongly that a final common pathway for signalling exists through which the proliferative actions of the CSFs become funnelled. However, the variety of other actions of the CSFs, many of which are unique to a particular CSF, and have different locations in the responding cells, indicates that CSF receptors must have the ability to initiate multiple signalling cascades impinging on a variety of locations in the cell.

6. SITES AND CONTROL OF CSF PRODUCTION

CSF of one type or another can be detected in the plasma, urine and extracts of all tissues tested (Metcalf 1984). There are emerging differences between the CSFs in this regard. M-CSF, at least in mice, is normally detectable in the plasma and, in man, always detectable in the urine. In contrast, G-CSF and GM-CSF are less frequently detected in normal plasma and have not been identified with certainty in the urine. To a large degree, the exact CSFs extractable from various organs have yet to be characterized. Multi-CSF is a particularly enigmatic molecule as it has not been detected *in vivo* in normal health.

Table 4. *Cell types able to produce colony-stimulating factors*

cell type	type of CSF produced	inducing signals
endothelial cells	GM-CSF, G-CSF, M-CSF	endotoxin, IL-1, IL-6, TNFα
stromal cells	M-CSF, GM-CSF, G-CSF	endotoxin, IL-1, IL-6
fibroblasts	M-CSF, GM-CSF, G-CSF	IL-1, IL-6, Endoxotin
macrophages	G-CSF, M-CSF, GM-CSF	endotoxin, M-CSF, Multi-CSF, γ-IFN
T-lymphocytes	GM-CSF, Multi-CSF	Foreign antigens, mitogens, receptor binding, IL-2, IL-1
B-lymphocytes	GM-CSF	mitogens
mast cells	Multi-CSF, GM-CSF	IgE receptor activation
uterine epithelial cells	M-CSF	pregnancy
keratinocytes	Multi-CSF	

All methods of estimation suggest that normally the rate of production of the CSFs and their resulting concentrations in the tissues are low under basal conditions, although levels can rise sharply in abnormal situations, such as responses to infections.

These general observations carry a number of implications. CSFs may in some instances behave like typical hormones but in other situations CSF production and action may occur locally and have no impact on circulating CSF levels. Furthermore, the content of CSF in multiple organs suggests either that the cell types producing CSF are widely distributed in the tissues or that multiple cell types must be able to produce one or more CSF.

It may seem surprising after 20 years of work that there could remain significant uncertainties regarding the cellular sources of the CSFs and the factors controlling their production. However, considerable uncertainties do exist and have several causes: (i) the low levels of CSF transcription and production are often below detectable levels using analysis by bioassay, Northern analysis, *in situ* hybridization and sometimes even the polymerase chain reaction; (ii) the occurrence of a profound induction of CSF transcription following manipulation of cells during attempts to purify the cell types under test or during culture of such tissues or cells; (iii) the general unreliability of data obtained from established cell lines, whether normal or neoplastic, and (iv) the technical difficulties preventing purification of most cell types in the body for subsequent testing. At least some cell types have the capacity to bind CSF to their cell surface, complicating efforts to establish the origin of the CSF (Gordon *et al.* 1987).

The consequences of these unresolved problems are that information on cells able to produce CSF has been restricted to a relatively small number of cell types able to be obtained readily in purified form. Even with these cells it is now recognized that *in vitro* inductive events may have led to serious overestimates, not of their capacity to produce CSF, but of their actual level of production under *in vivo* conditions.

To compound these problems it is now recognized that networks of regulators can interact with profound effects on the production of a particular CSF. As a consequence of this and the short half-life of CSF mRNA, the mere act of separating a cell population for study from other unwanted cell types may itself lead to serious distortion of the expressed function of the cells.

These negative comments on what is now a large body of published information show the need for considerable caution and scepticism in assessing the significance of the information.

What has been observed by using cultured cells is that multiple cell types can exhibit a capacity to produce CSF and that often a single cell type can simultaneously produce more than one CSF (table 4). CSF-producing cells include stromal cells, fibroblasts, T- and B-lymphocytes, macrophages, mast cells and endothelial cells, all of which are widely distributed

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throughout most organs. The contribution of these dispersed cell populations may be sufficient to account for the CSF able to be extracted from, or produced by, various organs *in vitro*.

This distribution of CSF-producing cells fits in well with the observations that the most potent natural inducing signals are products of microorganisms such as endotoxin or factors induced by such products, such as IL-1 or IL-6 (Metcalf 1984, 1988). It could therefore be envisaged that, following entry of microorganisms into the body, the first cells likely to be exposed to the products of such microorganisms are those equipped to respond by an enhanced production of CSF. Such an early infection might well be aborted by simple CSF-enhancement of the functional activity of existing granulocytes and macrophages. If the infection persisted, then microorganism products would be likely to enter the systemic circulation and to induce the generalized production of CSF throughout the body.

A more general extension of this concept would suggest that all cells in the body might have at least some capacity, when induced by appropriate signals, to produce CSF. This would not be an unreasonable proposition teleologically if the CSFs in essence are a signalling system designed to allow any cell in the body to make use of the specialized functions of granulocytes and macrophages to prevent or repair damage to that cell or its neighbours.

The most efficient inductive signals vary according to the responding cell type. Endotoxin, either directly or via the induction of IL-1, is a potent inducer of G-CSF, GM-CSF and M-CSF production by fibroblasts, stromal cells and endothelial cells (Bagby *et al.* 1983; Rennick *et al.* 1987; Seif *et al.* 1987; Zsebo *et al.* 1988) and this series of cells may well be the most important quantitative source of these CSFs in many circumstances. When primed *in vitro*, T-lymphocytes have a capacity to produce large amounts of GM-CSF, Multi-CSF (and in the case of human cells, M-CSF) as well as certain other growth factors such as IL-4 and IL-5 (Kelso & Metcalf 1990). B-lymphocytes also have some ability to produce CSF (Pistoia *et al.* 1987). It remains uncertain whether these potential abilities of lymphocytes are expressed in the normal body. The inductive signals required for activation of lymphocytes involve either exposure to foreign antigens or other methods for cross-linking the T- and B-lymphocyte surface receptors. Activation of this type has been documented *in vivo* in the exceptional situation of graft-versus-host disease after transplantation of partially mismatched marrow cells and should in principle also occur in a variety of infections and other disease states associated with lymphocyte activation.

A special situation can exist in certain types of cancer where the cancer cells themselves express a marked ability to produce CSF. Because resection of such tumours is followed by a fall in systemic CSF levels, it can be presumed that in such patients, the tumour made a significant contribution to total body CSF levels.

The molecular basis of transcription induction for the CSFs has been reviewed by Gough & Nicola (1990). Typical binding sites for specific nuclear transcription factors have been identified upstream of the CSF genes and some progress has been made in isolating these transcription factors. As is the case for other genes of this type, it is likely that an extremely complex series of molecular interactions is involved in transcriptional regulation.

What is clear from the biology of CSF production by cells is that the induction systems allow individual cells to synthesize more than one regulator simultaneously and levels of mRNA and protein production to be altered radically within hours (Kelso & Metcalf 1990). This represents a highly efficient system for responding quickly to sudden emergencies such as the onset of a potentially serious infection. However, as with the redundancy evident from the existence of multiple regulatory factors, it is again puzzling why, as in the case of T-lymphocytes, it should be advantageous for an individual cell to produce as many as five or six regulators.

7. *IN VIVO* EFFECTS OF THE CSFs IN EXPERIMENTAL ANIMALS

The *in vitro* studies on CSF action predicted strongly that CSFs should be able to influence the production and function of granulocytes and macrophages when injected *in vivo*.

Logistical problems delayed for almost a decade experiments to determine the *in vivo* effects of injected CSF. Studies using trace amounts of CSF showed that, in keeping with other glycoprotein regulators, the CSFs had relatively short half-lifes in the plasma after intravenous injection (typically in the 1–3 h range) but that more sustained elevations could be achieved by subcutaneous or intraperitoneal injections (Metcalf *et al.* 1986*a*, 1987*b*).

For the reasons discussed earlier, the only feasible approach to studying the *in vivo* responses to injected CSF, even in mice, required the use of recombinant CSF. Experience with erythropoietin had shown the necessity for using a glycosylated form if responses were to be achieved *in vivo*, but this proved not to be necessary for the four CSFs. After the demonstration that recombinant CSFs had the same actions and specific activity *in vitro* as native CSFs (Metcalf *et al.* 1986*b*, 1987*a*; Souza *et al.* 1986), the way was clear to begin studies on the responses in mice to injected purified recombinant murine CSFs. The use of the intraperitoneal route was preferred in this laboratory because it provided an opportunity to compare systemic responses, as evident in the blood, marrow or spleen with local responses, as monitored in the peritoneal cavity injection site.

Given the potential complexity of the situation *in vivo*, with the likely existence of interacting regulatory cascades and negative feedback systems that might suppress any induced perturbation, it proved surprisingly easy to induce does-related changes in haemopoietic populations in mice by injecting CSF.

Elevations were observed in blood levels of neutrophils in mice injected with GM-CSF or G-CSF and both neutrophils and eosinophils in the case of mice injected with Multi-CSF. With the doses used, G-CSF

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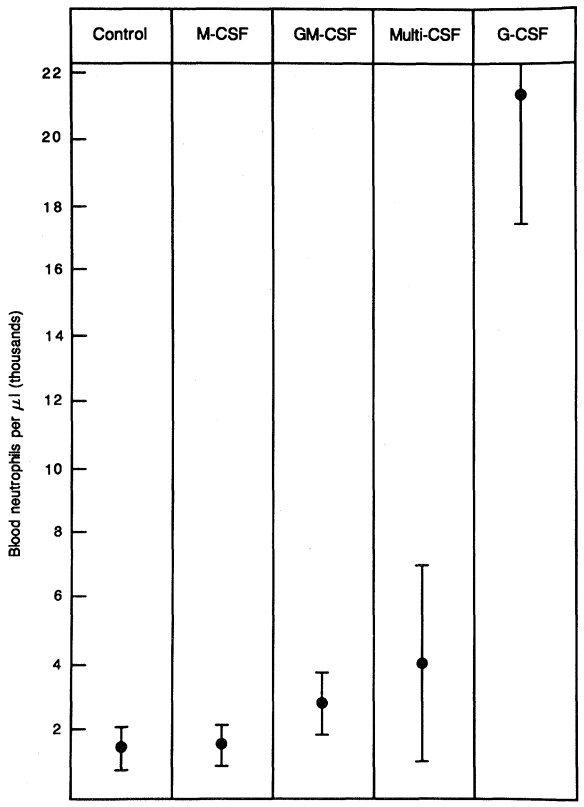

Figure 5. Levels of neutrophils in the blood of adult BALB/c mice after 6 d of injection of 100 ng CSF, three times daily. Note that G-CSF is the most active CSF in elevating blood neutrophil levels. Mean values ± standard deviations of 8–12 mice per group.

was by far the most effective CSF in elevating neutrophil levels, the cells being almost entirely mature neutrophils with only a low percentage of less mature forms (figure 5) (Metcalf *et al.* 1986*a*, 1987*b*; Moore & Warren 1987; Tamura *et al.* 1987; Molineux *et al.* 1990).

In mice, acute elevations compatible with the release of pre-existing neutrophils from the marrow have been noted (Hattori *et al.* 1990), although this action is more obvious in man. The progressive rise in neutrophils with continuing CSF injections is consistent with an increased production of cells in the marrow, with near-normal maturation and release times.

The bone marrow in the mouse is normally fully occupied by cells and no agent can be expected to increase the number of marrow cells. In mice, the spleen is normally the site of some haemopoiesis, albeit usually at a low level. Where increases in total haemopoietic cells occur in the mouse, the additional haemopoietic cells accumulate in the spleen, resulting in spleen enlargement. None of the CSFs induced rises in marrow cell numbers and indeed GM-CSF induced a slight fall in total cell numbers. However, each CSF induced a rise in the percentage of granulocytic or monocytic cells in the marrow. As in the case of blood cell rises, G-CSF elicited the greatest rise in percent granulocytic cells. All four CSFs induced spleen enlargement, with again G-CSF eliciting the largest rise following by Multi-CSF then GM-CSF and M-CSF.

The liver is the other organ in the mouse that can develop haemopoietic populations in situations in which haemopoiesis is generally increased. In mice injected with all four CSFs, a rise was observed in granulocytic and macrophage cells in the liver. However, it was exceptional to observe mitotic activity in such cells and the liver may merely have been acting as an initial repository for additional cells produced in the marrow and spleen.

While the cell types responding *in vivo* faithfully paralleled those known to respond to the various CSFs *in vitro*, the dominance of G-CSF in eliciting systemic responses was unanticipated. *In vitro*, G-CSF induces the formation of relatively low numbers of quite small granulocytic colonies, particularly in comparison with the more numerous and larger colonies stimulated by GM-CSF or Multi-CSF.

The basis for this quantitative reversal of the anticipated hierarchy of CSF molecules has yet to be established. To a degree, the results in mice proved to be slightly misleading in that subsequent studies in primates and man with comparable CSF doses did reveal a more obvious ability of GM-CSF and Multi-CSF to elicit rises in granulocyte levels than was apparent in the mouse. A distinct difference remains, however, even in man, because with comparable doses G-CSF elevates neutrophils in the blood to higher levels than does GM-CSF or Multi-CSF. Mice are capable of exhibiting gross elevations of granulocytes in response to GM-CSF or Multi-CSF when much higher concentrations of CSF are achieved. Thus, in mice repopulated by haemopoietic cells producing GM-CSF or Multi-CSF, where CSF concentrations in the plasma can reach 10^5 Units ml^{-1}, granulocyte levels can reach $1–3 \times 10^5$ per microlitre (Johnson *et al.* 1989; Chang *et al.* 1989*b*). Mice appear merely to be relatively unresponsive, rather than refractory, to stimulation by GM-CSF or Multi-CSF as assessed by blood-cell responses.

A quite different situation was observed in cellular responses elicited by the CSFs at the local site of injection in the peritoneal cavity. In this location, GM-CSF proved to be the most highly active agent and induced major rises in peritoneal macrophage numbers, with substantial rises also in neutrophil and eosinophil numbers (Metcalf *et al.* 1987*b*). Responses to other CSFs were observable but were lower in magnitude (figure 6). The rise in peritoneal macrophage populations elicited by GM-CSF was not due simply to a locally induced migration of cells from the circulation to the cavity. A dose-related rise in mitotic activity was observed in peritoneal macrophages in mice injected with GM-CSF that was sufficient to account for much of the rise in total cell numbers (Metcalf *et al.* 1987*b*). Furthermore, although the responses were quantitatively smaller, subcutaneously injected GM-CSF was also able to induce rises in peritoneal cavity populations.

These observations raise the possibility that distinct differences exist in the biological actions of the CSFs. For the two prototype CSFs – G-CSF and GM-CSF – it may be that G-CSF is an agent with a dominant action on systemic granulocytic populations, whereas

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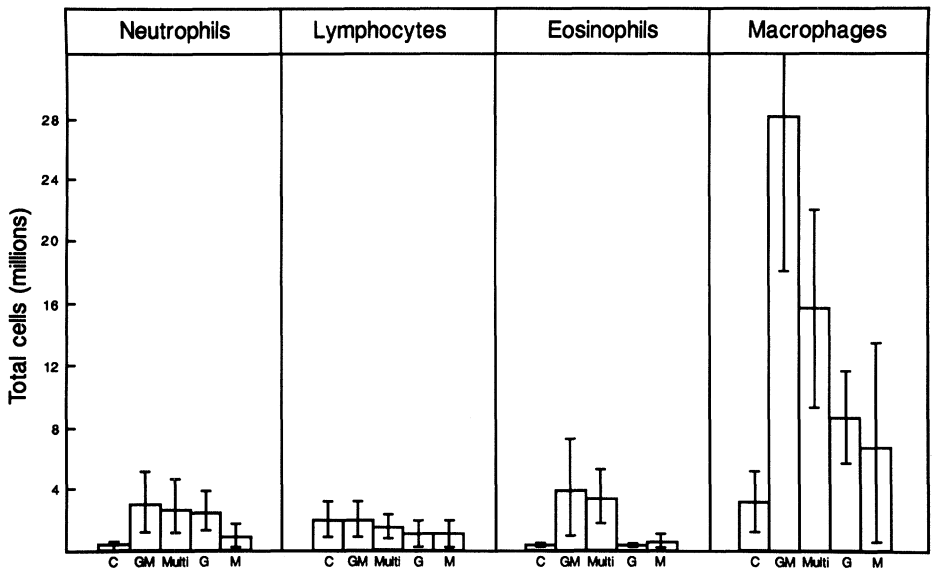

Figure 6. Total numbers of cells in the peritoneal cavity of adult BALB/c mice after 6 d of intraperitoneal injections of 100 ng CSF, three times daily. Mean values ± standard deviations of eight mice per group. Note that GM-CSF is the most active CSF in elevating numbers of macrophages, neutrophils and eosinophils.

GM-CSF is an agent having its most powerful effects on local granulocyte–macrophage populations.

These studies suggest that there may be distinct differences between the biology and control of systemic versus local granulocyte–macrophage populations and that reliable information on the behaviour of these cells in local lesions may not be able to be obtained clinically, simply by measuring the levels of blood cells or circulating regulatory molecules.

These conclusions are strongly supported by the changes developing in GM-CSF transgenic mice in which GM-CSF levels are elevated at least 100-fold throughout life. Such mice exhibit normal numbers of granulocytes and monocytes in the peripheral blood, normal cellularity in the marrow and spleen, and normal numbers of progenitor cells in these tissues. In sharp contrast, the numbers of macrophages, neutrophils and eosinophils are massively elevated in the pleural and peritoneal cavities and in certain tissue locations (Lang *et al.* 1987).

It is of interest that, if G-CSF is combined with GM-CSF, then the responses observed are not only superadditive, as would have been anticipated from *in vitro* studies, but that the distinctive elements of each response are retained. Specifically, the elevation of blood neutrophil levels is retained, as is the elevation of peritoneal macrophage numbers.

In agreement with *in vitro* observations showing that GM-CSF and Multi-CSF also have actions on cells outside the granulocyte–macrophage lineage, mice injected with high doses of GM-CSF also developed rises in eosinophils and in spleen megakaryocytes (Metcalf *et al.* 1987*b*). Similarly, Multi-CSF elicited rises in eosinophil levels and a major rise in megakaryocytes, particularly in the spleen. Multi-CSF is the only CSF with proliferative actions *in vitro* on mast cells; rises in mast cell numbers were observed only in mice injected with Multi-CSF and involved mast cells both of tissue type as seen in the subcutaneous tissues and of the T-lymphocyte-dependent type. Indeed, rises in the latter type of mast cell were marked in the spleen (up to 100-fold) and proved to be the most sensitive index of responsiveness to the injection of Multi-CSF (Metcalf *et al.* 1986*a*, 1987*a*).

Committed progenitor cells are not capable of self-renewal, *in vitro*, and become expended in response to the CSF-stimulated formation of maturing progeny. Progenitor cells are generated by the proliferative activity of more ancestral cells in the stem cell compartment. *In vitro* tests, using individual CSFs suggested that G-CSF and M-CSF have no direct proliferative actions when acting alone on multipotential stem cells but that Multi-CSF exhibited moderate activity and GM-CSF had the capacity to stimulate the proliferation of at least some multipotential precursors (Metcalf 1984). However, combination of CSFs with other agents active on stem cells such as interleukin-1 or stem cell factor results in the considerably enhanced formation of progenitor cells (Moore & Warren 1987; Zsebo *et al.* 1990).

It was not entirely predictable therefore whether the administration of CSF *in vivo* might lead to a self-terminating response when all existing progenitor cells had been depleted, or whether the injected CSF would actually increase progenitor cell numbers, either by direct action or by interaction with other regulatory factors in the recipient. What was observed was that CSF-stimulated responses did not abruptly diminish and could be sustained for as long as CSF injections were continued, although the periods studied in mice were usually only of one to two weeks' duration. Instead of a fall occurring in stem or progenitor cell numbers, the CSFs in fact elicited rises in the numbers of such cells and again, somewhat surprisingly, G-CSF proved to be the most active agent (Metcalf *et al.* 1986*a*, 1987*b*; Moore & Warren 1987; Molineux *et al.* 1990).

Two types of response were observed in CSF-injected mice that could not have been predicted from *in vitro* studies because they involved population

redistributions in the body. In the first, pronounced rises were observed in blood levels of progenitor and stem cells, a site normally containing few such cells. The second curious response, noted most dramatically with G-CSF, was a major redistribution of erythropoietic populations from the marrow to the spleen. Normally the mouse marrow contains more than 95% of the nucleated erythroid cells in the body and the spleen less than 5%. However, with seven days of G-CSF injections, this situation was reversed. The marrow content of erythropoietic cells was severely reduced and in the enlarged spleen of such animals more than 60% of the cells were immature erythroid cells. A more minor rise in spleen erythroid cells was also observed after the injection of Multi-CSF and GM-CSF, but this was less surprising, given the ability of Multi-CSF, and to a lesser degree GM-CSF, to stimulate the proliferation of erythroid cells *in vitro*.

The mechanisms responsible for these population redistributions have not been characterized, but they may be the consequence of interactions between the CSFs and other pre-existing regulators *in vivo* or the induction by the CSFs of the production of agents able to elicit these remarkable phenomena.

The injection of CSF did not result in any apparent toxic effects in the mice nor were histological changes observable in any organ other than those involving haemopoietic populations.

It was of interest to determine whether CSF had elicited functional activation of mature neutrophils and monocyte–macrophages in the injected animals. Tests using macrophages from mice injected with GM-CSF or Multi-CSF documented an activation of phagocytic capacity comparable with that achievable *in vitro* (Metcalf *et al.* 1986*a*, 1987*b*).

The conclusions from these initial *in vivo* studies were that the CSFs are active *in vivo* and able to stimulate the formation and function of granulocytes and macrophages in a manner comparable with their action *in vitro*. The data from various laboratories strongly suggested therefore that the CSFs are regulators of major importance for these populations *in vivo*.

It is proper, however, to question this conclusion. Granted that the CSFs are active *in vivo*, does this really prove that the CSFs are the usual, or most important regulators of granulocytes and macrophages *in vivo*, particularly under basal conditions? Criticisms had been raised on this latter question throughout the developmental work on the CSFs because the CSFs were most readily detectable during states of perturbation, such as during infections or following the injection of endotoxin. It was argued that the CSFs might merely be molecules used by the body in emergency situations and that the mechanisms controlling the basal production of granulocytes and monocytes might be quite distinct. Because of the highly restricted distribution of haemopoietic cells and the evidence for a local, and possibly cell-contact, regulatory role played by marrow stromal cells, such criticisms went further and proposed that normal granulocyte–macrophage formation might be stromal-controlled by mechanisms that did not involve the CSFs.

There are certain general arguments that can be raised to counter such criticisms. Studies have now shown that stromal cells either constitutively produce CSF (in the case of M-CSF) or can readily be induced by physiological signals such as IL-1 to produce CSF (in the case of G-CSF and GM-CSF) (Gualtieri *et al.* 1987; Rennick *et al.* 1987). Furthermore, such M-CSF can be displayed in a membrane-bound form that is functionally active. Stromal cells do produce other regulatory molecules such as IL-6, IL-7, SCF and LIF and may well produce additional molecules yet to be detected. Some of these do have direct or indirect actions influencing the formation of granulocyte–macrophage populations.

The fact remains that, to date, no agents other than the CSFs, IL-6 and SCF have been detected in any tissue-derived material, including marrow-stromal-cell-conditioned media, that are able to stimulate granulocyte–macrophage colony formation. Furthermore, in the studies eventually leading to the purification of the CSFs from a variety of tissue-conditioned media no active fractions were knowingly discarded that might have contained additional factors. The much higher concentrations of SCF or IL-6 needed to elicit granulocyte colony formation *in vitro* than is the case for the CSFs, make it less likely that SCF or IL-6 play a dominant role *in vivo*.

While such arguments have validity, they are essentially negative in nature and can only lead to provisional conclusions. To prove that the CSFs play a key role in the control of basal haemopoiesis requires experiments in which suppression of CSF has been achieved *in vivo* and results in some type of reduction in granulocyte–macrophage populations. Given the overlapping actions of the CSFs, this presents some difficulties, but evidence could be obtained by the use of CSF antibody mixtures or the development of mice in which CSF genes have either been deleted or shut off.

Evidence of this type has now been obtained to substantiate a role for at least two of the CSFs. In dogs injected with human G-CSF, the CSF is sufficiently antigenic to elicit the formation of neutralizing antibodies. However, human and canine G-CSF are sufficiently similar for these antibodies to be cross-neutralizing. In this situation, the dogs exhibit a reduction of normal neutrophil levels, implying strongly that G-CSF was required for maintenance of normal neutrophil levels (Hammond *et al.* 1991).

An interesting genetic anomaly in mice has been described (the osteopetrotic mouse, op/op) in which macrophage numbers are defective, as is the formation of their derivative osteoclasts. In the absence of the normal bone remodelling function of these osteoclasts, the marrow cavity becomes obliterated by excess osseous tissue. The gene responsible for the osteopetrotic defect is located on chromosome 3 and the M-CSF gene has been mapped to the same region. Sequence analysis has shown that the M-CSF gene in op/op mice contains a nonsense mutation at the codon for amino acid 277 preventing transcription of the 2.3 kilobase (kb), M-CSF mRNA and M-CSF production. Introduction of M-CSF into op/op mice has at least

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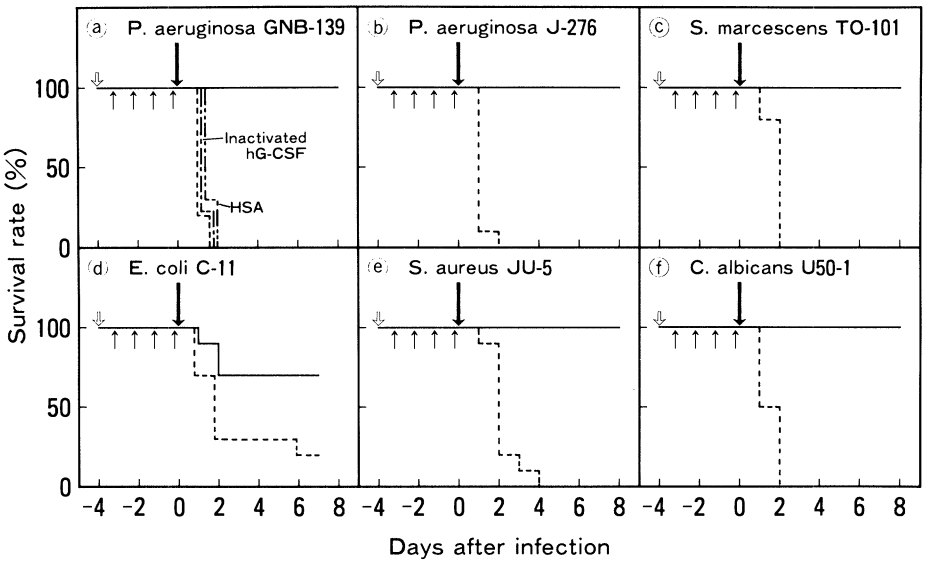

Figure 7. In mice with haemopoietic damage induced by the cytotoxic drug cyclophosphamide (open arrow), pre-injection with G-CSF protects the mice from challenge with a lethal dose of a variety of organisms (solid arrow). Unbroken line shows survival of G-CSF-injected mice. Reproduced with permission from Matsumoto *et al.* (1987).

partially corrected their defects (Wiktor-Jedrzejczak *et al.* 1990). By implication, this model indicates that M-CSF is necessary for normal macrophage and osteoclast production and function.

The ability of injected CSF to induce an increased production of granulocytes and macrophages and their functional activation, although by itself of great interest, is only of practical importance in clinical medicine if the CSF-induced responses in fact lead to a demonstrable increase in resistance to infections.

Several studies have now shown that the administration of CSF does significantly enhance resistance and decreases mortality in experimental animals. In mice rendered hypersusceptible by prior administration of the myelotoxic drug cyclophosphamide, pre-injection of the animals with G-CSF increased resistance approximately 1000-fold to challenge with otherwise lethal doses of microorganisms ranging from pseudomonas to candida (figure 7) (Matsumoto *et al.* 1987). Similar enhanced resistance has been observed in rat and rabbit model infections. Mortality from whole-body irradiation of conventional mice has as a major component an induced susceptibility to lethal infections. In mice subjected to lethal doses of whole-body irradiation, subsequent injection either of GM-CSF or G-CSF resulted in a substantial increase in survival (Talmadge *et al.* 1989; Tanikawa *et al.* 1990). Similarly, the CSFs have been found to synergize with other agents such as IL-1 or tumour necrosis factor in conferring radioprotection (Neta *et al.* 1988).

Following this initial work in mice, studies in primates using human CSFs confirmed that the CSFs were capable of elevating blood and marrow levels of granulocytes and monocytes using comparable doses to those used in mice and that such responses were also able to be elicited in animals whose marrow had been damaged by prior use of cytotoxic drugs (Donahue *et al.* 1986; Welte *et al.* 1987; Mayer *et al.* 1987; Nienhuis *et al.* 1987). As in the case of mice, these responses were achievable without evidence of serious toxic effects. Following cessation of CSF injections, levels of granulocytes and monocytes returned to pre-injection levels within several days.

8. CLINICAL TRIALS OF THE CSFs

The objectives of the initial clinical studies were to determine the toxicity of the injected CSF and its ability to produce significant changes in granulocytic or monocyte populations in the recipients. To date, most patient groups studied have had subnormal haemopoiesis either as a direct consequence of a disease state such as AIDS, myelodysplasia, congenital or cyclic neutropenia or where haemopoietic populations had been damaged following chemotherapy for cancer.

Initial studies showed that subcutaneous injections are usually the most effective method for administration of CSF (Cebon *et al.* 1990). This has permitted self-administration of CSF by patients using daily subcutaneous injections.

It was established that G-CSF exhibited minimal toxicity and that GM-CSF, at doses below 10–15 μg kg^{-1} d^{-1}, exhibited only minor levels of toxicity. With higher doses of GM-CSF, some serious adverse responses were encountered such as the capillary leak syndrome or pericarditis.

Both G-CSF and GM-CSF proved capable of elevating blood granulocyte levels in a dose-dependent manner. Following injection of either CSF there was a brief period of leukopenia due to CSF-induced adherence of circulating cells to vascular endothelium. This was followed by a progressive rise in blood granulocyte levels, with the marrow showing increased cellularity and a rise in the proportion of granulocytic cells at various stages of differentiation. This response was able to be maintained for as long as daily CSF administration was continued. After cessation of CSF injections, white-cell levels return to pre-injection levels within 1–3 d. GM-CSF was also capable of elevating blood monocyte and eosinophil levels (Bronchud *et al.*

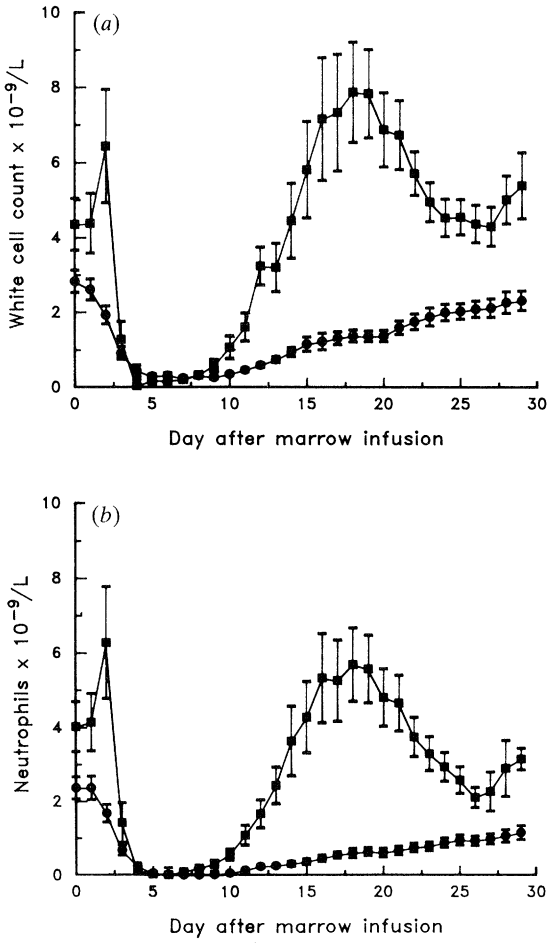

Figure 8. In patients subjected to chemotherapy then given an autologous bone marrow transplant, the injection of G-CSF accelerates the regeneration of blood neutrophil levels. The upper curve in each panel shows levels in 15 G-CSF-injected patients compared with levels in an historical control series of 18 patients. Reproduced with permission from Sheridan *et al.* (1989).

1987; Groopman *et al.* 1987; Morstyn *et al.* 1988; Gabrilove *et al.* 1988; Lieschke *et al.* 1989). Parallel studies produced evidence, as in the mouse, that CSF injections could result in functional activation of granulocytes and monocytes as measured by parameters such as superoxide production or cytotoxicity (Kaplan *et al.* 1989; Wing *et al.* 1989).

This basic pattern of responses was observable in all types of patient, although where the existing marrow was markedly aplastic, the magnitude of the observed responses was correspondingly reduced and in some patients with aplastic anaemia or severe myelodysplasia no significant responses were able to be elicited.

In general therefore, humans proved to be as responsive to CSF injections as mice or primates and responses were observable even where the marrow had sustained a certain level of damage due to disease or cytotoxic therapy.

On the basis of these initial clinical data, more complex trials were undertaken to determine whether CSF administration could (i) significantly accelerate granulocytic regeneration after chemotherapy or marrow transplantation (Bronchud *et al.* 1987; Brandt *et al.* 1988; Gabrilove *et al.* 1988; Morstyn *et al.* 1988; Neumanitis *et al.* 1988; Sheridan *et al.* 1989) or (ii) increase granulocyte levels in patients with congenital or cyclic neutropenia or myelodysplasia (Vadhan-Raj *et al.* 1987; Yuo *et al.* 1987; Antin *et al.* 1988; Bonilla *et al.* 1989; Ganser *et al.* 1989; Hammond *et al.* 1989; Kobayashi *et al.* 1989; Thompson *et al.* 1989; Negrin *et al.* 1990; Welte *et al.* 1990). The results of these trials indicated that CSF administration can significantly accelerate haemopoietic regeneration. For example, following autologous marrow transplantation in cancer patients, recovery of neutrophil levels to more than 1000 per microlitre was able to be accelerated by up to 7–10 days (figure 8), with shortening of the period of intensive nursing (Neumanitis *et al.* 1988; Sheridan *et al.* 1989).

The ability of injected CSF to accelerate haemopoietic regulation after marrow transplantation has interesting basic implications for the effectiveness of the regulatory systems normally controlling haemopoiesis. It might have been anticipated in a patient with an aplastic bone marrow, and often with an infection, that a maximal possible stimulation of haemopoiesis would occur following activation of the complex induction systems controlling the production of regulators. The ease with which injection of a single regulator alters the rate of regeneration implies strongly that, even under extreme circumstances, the body cannot mount a maximal regulatory response. If so, the administration of such regulators serves a useful function, and this conclusion presumably applies also for a number of haemopoietic regulators in addition to the CSFs.

In congenital and cyclic neutropenias, administration of G-CSF has been successful in elevating neutrophil levels above 1000 per microlitre and, with daily CSF injections, in sustaining such elevations indefinitely (Bonilla *et al.* 1989; Hammond *et al.* 1989; Welte *et al.* 1990). Patients with cyclic neutropenia have now been under CSF therapy for up to three years without loss of responsiveness to CSF or the development of neutralizing antibodies.

As with the studies in animals, the question at issue in such treatment procedures is not simply whether granulocyte levels can be elevated but whether this leads to a significant reduction in the susceptibility of the patients to infections or to a resolution of pre-existing infections. Most studies undertaken so far have documented some level of enhanced resistance to infections. This increased resistance is certainly not absolute, for in all studies on CSF-treated patients, some examples were encountered of infections developing during CSF therapy. The clearest evidence so far produced to show that CSF treatment results in a clinically valuable increase in resistance has come from studies on patients with congenital or cyclic neutropenia. In such patients, the reduction in infections is so unequivocal that chronic CSF therapy seems established as an adequate substitution therapy.

With the more common clinical situation of cancer patients at risk of infections after intensive chemotherapy, the situation is less easy to document because of the heterogeneity of this patient group and a variety of double-blind trials will be needed to establish the

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level of resistance achievable. There are numerous anecdotal cases where CSF treatment has been successful in eliminating intractible antibiotic-resistant infections but, although tantalizing, these cases do not provide a sound basis on which to base future treatment procedures.

In patients injected with either G-CSF or GM-CSF, up to a 100-fold rise occurs in progenitor cells of all lineages in the peripheral blood to levels approaching those in the marrow (Dührsen *et al.* 1988; Socinski *et al.* 1988; Villeval *et al.* 1990). Although the basis of this phenomenon remains unresolved, the response is dramatic and has raised the use of peripheral blood cells harvested from CSF-pre-injected patients as an alternative source of repopulating cells to bone marrow for autologous transplantation of particular value because of the more rapid repopulating ability of cells from the peripheral blood.

For patients receiving chemotherapy or marrow transplantation, an unsolved clinical problem is the associated thrombocytopenia that still requires hospitalization and platelet transfusions. The CSF with the most obvious ability to stimulate megakaryocyte formation is Multi-CSF, and in current clinical trials Multi-CSF is being examined alone or in combination with other CSFs to determine whether it will achieve a useful acceleration of regeneration of platelet levels (Ganser *et al.* 1990).

M-CSF is also in early clinical trial and may prove of particular value in clinical situations requiring macrophage stimulation. In mice, M-CSF administration has improved osteopetrosis by enhancing osteoclast formation and its use in the analogous human disease will be followed with interest. It is less clear whether some types of infection require macrophages as a special feature of the host response. It has been somewhat surprising to discover the diversity of bacterial and fungal infections that appear to be influenced by the injection of G-CSF where there is a selective granulocyte response, but it must be presumed that certain infections do require the special intervention of macrophages.

Studies *in vitro* and in experimental animals have shown that combinations of CSF elicit enhanced proliferative responses that can be markedly superadditive. The use of CSF combinations has potential clinical advantages in reducing the doses of each required and thus in minimizing the risk of adverse reactions, while at the same time either broadening the range of responding cells or the location of cellular responses. There will also be situations where it is advantageous to combine CSF therapy with the administration of other haemopoietic regulators.

A special situation exists with patients with myeloid leukaemia. Virtually all leukaemic populations in such patients remain dependent on, and are responsive to, proliferative stimulation by the CSFs (Metcalf 1984). No substantial difference has been noted between the different CSFs in this regard, although M-CSF may have somewhat weaker proliferative actions. The use of a CSF to stimulate haemopoiesis in a myeloid leukaemic patient might therefore seem to be highly undesirable because it would carry the risk of amplifying the leukaemic population. However, the situation is more complex as the differentiation commitment action of the CSFs on myeloid leukaemic cells can potentially result in complete suppression of a susceptible leukaemic population.

In situations where the leukaemic population has been eliminated by cytotoxic therapy, with or without marrow transplantation, the use of CSF therapy carries little risk and could be anticipated to have the same general value as in patients with other types of cancer where myelosuppression has followed chemotherapy.

Some cytotoxic agents used in the treatment of myeloid leukaemia have an action that is restricted to actively cycling cells. The possibility is being tested clinically that the use of CSF to force a higher-than-usual proportion of leukaemic cells to enter active cell cycle will induce a heightened susceptibility of the leukaemic population to destruction by cycle-specific agents.

There are therefore certain situations in which CSF therapy, either alone or in combination with other regulators, should be of value in the management of myeloid leukaemia.

There are many patients in whom haemopoiesis appears to be relatively normal yet the patients either have difficulty eliminating infections or are at known risk of acquiring a serious infection. These include the miscellany of patients who have a serious infection complicating some other disease state, such as diabetes. More definable are patients having gastrointestinal or urinary-tract surgery or with trauma or severe burns where infections are not uncommon sequelae, and patients with chronic or recurring infections such as urinary-tract infections. If the CSFs prove to be as nontoxic as presently appears, the more general therapeutic or even prophylactic use of CSFs for many of these patients would be reasonable.

Although one hesitates to envisage the possibility of future radiation accidents or, worse, of the use of atomic weapons, the CSFs are stable on storage and have been shown to be effective in reducing post-irradiation mortality in experimental animals. GM-CSF has already been used to accelerate haemopoietic regeneration in persons exposed in the Brazil radiation accident (Butturini *et al.* 1988). It could be proposed therefore that CSF stockpiles should be accumulated nationally for possible use in disasters of this type or in future pandemic infections.

At the present time it is unclear for some disease states how long CSF treatment needs to be continued. It seems likely that two general situations will emerge: relatively short courses of CSF treatment on a hospital or outpatient basis, for patients with severe infections or regenerating haemopoietic populations and, for a less-numerous group of patients, the prospect of lifetime self-treatment analogous with the situation of insulin self-administration by diabetics.

9. POTENTIAL HAZARDS OF CSF THERAPY

If differentiation commitment of multipotential haemopoietic stem cells is able to be induced by regulator action, a proposition still awaiting unam-

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biguous documentation, it is theoretically possible that the sustained use of one particular regulator would so perturb the commitment of stem cells in forming various progenitor cells that a severe depletion of progenitor cells for certain lineages might result.

In general, such lineage depletions have yet to be observed in patients receiving erythropoietin or in the majority of patients receiving G-CSF or GM-CSF. It would appear therefore that the reserve of stem cells is usually sufficient to maintain progenitor cell production in multiple lineages or that these regulators are not able to behave in a dominant manner *in vivo* to force lineage-restricted commitment.

A potential problem is posed by the ability of CSFs to stimulate the functional activity of mature cells, particularly macrophages, with the production by these cells of products that are potentially damaging to the tissues. In general, adverse reactions to moderate doses of CSFs are readily controllable and it suffices to be aware of the likely basis for such toxic effects.

It has become appreciated that while CSFs are quite capable of stimulating elevations of granulocytes and macrophages to extremely high levels, e.g. 100000 per microlitre, there is unlikely to be any additional benefit, in terms of increased resistance, from elevating cell counts to such unreasonably high levels. With this more rational approach to therapy, and the consequent use of lower doses of CSFs, the problem of the excess functional stimulation of mature cells should be minimal.

A much-discussed potential risk of CSF treatment has been the possible induction of myeloid leukaemia. Extensive work in animal models has shown that excess stimulation of cell proliferation in granulocyte–macrophage populations, even at extreme levels, does not result in leukaemic transformation if the target cells were otherwise normal (Metcalf & Moore 1988; Johnson *et al.* 1989; Chang *et al.* 1989*a*,*b*). On this basis, CSF administration could not be considered as carrying any significant risk of inducing myeloid leukaemia.

A different situation exists with cells that have acquired an abnormal capacity for self-generation (Metcalf 1989*a*). Studies using murine CSF-dependent cells of this type have shown that after injection into animals, such cells seed in various locations where they presumably are maintained by local CSF-producing cells. After some months, leukaemic transformation can occur in such cells and the frequency of leukaemic transformation events appears to correlate with the total number of resident cells (Dührsen & Metcalf 1988, 1989). These experiments document a situation in which leukaemic transformation can occur in a previously abnormal population with a frequency that correlates with the total number of CSF-dependent cells at risk. In a clinical setting, this situation may exist in certain myelodysplastic patients who are known to be at heightened risk of myeloid leukaemia development. It is conceivable that if CSF was used to increase the formation of granulocytes and macrophages in such patients, the CSF might also lead to expansion of abnormal cell populations in such patients, thus indirectly increasing the risk of emergence of a leukaemic clone. These patients are currently receiving CSF therapy with overall benefit in terms of improved haemopoiesis, so that situation needs careful monitoring to establish whether an increased risk of leukaemia development might ultimately outweigh the more immediate benefits of CSF treatment.

10. DISEASE STATES INDUCED BY EXCESS CSF LEVELS

Several murine model systems have been developed to determine the pathological consequences of states in which CSF levels are greatly elevated. At least in mice, some distinctions seem to be emerging between the four CSFs that may be based on the ability or not of a particular CSF to stimulate macrophage function.

In transgenic GM-CSF mice, levels of GM-CSF are elevated at least 100-fold in the plasma and tissue fluids. Such mice develop a characteristic syndrome in which the marrow, spleen and blood haemopoietic populations appear unaltered but in which massive accumulations of macrophages and to a lesser degree neutrophils and eosinophils develop in the peritoneal and pleural cavities and certain tissues. The mice exhibit ocular opacity due to macrophage infiltration of the eye, with damage to the retina, but appear otherwise in good health for the first two months of life. Thereafter, the mice die prematurely often with paralysis or cachexia and with focal inflammatory lesions in the muscles and certain other tissues (Lang *et al.* 1987; Metcalf & Moore 1988). A more acute version of this syndrome can be induced by repopulating mice with bone-marrow cells producing very high levels of GM-CSF following retroviral insertion into the marrow cells of the GM-CSF cDNA under a strong retroviral promoter (Johnson *et al.* 1989).

The present belief is that the tissue lesions are induced by GM-CSF-stimulated macrophage production of excessive amounts of agents such as interleukin-1, tumour necrosis factor, γ-interferon and plasminogen activator that are toxic for adjacent tissues.

Comparable repopulation models, where mice have excess levels of Multi-CSF, exhibit massive haemopoietic hyperplasia and excess mast-cell numbers, with some shortening of the life-span of the animals (Chang *et al.* 1989*b*). In contrast, repopulated mice with grossly elevated G-CSF and granulocyte levels do not develop tissue lesions and do not exhibit a shortened survival (Chang *et al.* 1989*a*). The implications from mice of this latter type are that excessive stimulation of granulocytes may not lead to tissue damage.

These various observations have identified certain disease states in man in which excess levels of CSF might be involved in their pathogenesis. The disease state most closely resembling GM-CSF transgenic mouse is malignant histiocytosis of young children, where the lesions have a remarkable histological similarity to those in GM-CSF transgenic mice.

It will also be worthy of investigation whether a variety of chronic diseases, ranging from polymyositis and sarcoidosis to rheumatoid arthritis, may also

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involve as an aetiological component an excess production of GM-CSF or possibly M-CSF. Here, the biology of CSF production needs to be taken into account for the lesions may be associated with localized excess levels of CSF production that may not necessarily result in elevated plasma CSF levels.

11. SOME GENERAL BIOLOGICAL CONSIDERATIONS

The regulation of granulocyte–macrophage formation and function has proved to be unexpectedly complex, with four CSFs controling the behaviour of committed granulocyte–macrophage progenitor cells and their progeny. At least four other regulators, IL-6, SCF IL-4 and LIF, also have some actions on these populations.

If the regulatory control of granulocyte–macrophage populations is typical of the situation with the other major haemopoietic lineages, then an equally complex series of regulators can be presumed to exist for these other lineages, with many regulators still to be detected. When it is also considered that a complex network of other regulatory molecules can operate on cells able to produce these haemopoietic regulators, a pessimistic view might suggest that the whole control system of haemopoiesis is impossibly complex and does not allow the likelihood of useful intervention to control pre-existing abnormalities. However, it is obvious from clinical experience with erythropoietin and the CSFs that the use of even a single regulator can have spectacular and sustainable effects that are in substantial agreement with expectations from simple *in vitro* cultures. This implies that although complex networking of regulatory factors may exist, the net outcome of the interactions is not particularly powerful *in vivo* and is easily overridden by the injection of a single regulator.

A question which has arisen so far only to a minor degree with the CSFs is how genuinely selective are the actions of these agents, currently regarded as haemopoietic growth factors? What has forced this question into prominence is the situation with two regulators, IL-6 and LIF, that were originally purified as likely haemopoietic regulators but have subsequently been recognized to have striking actions on an almost bizarre spectrum of cells, ranging from hepatocytes to neuronal cells (Kishimoto 1989; Metcalf 1991).

Assessment of the cellular specificity of a regulator is not helped necessarily by determination of cell types expressing receptors for the molecule, because the presence of receptors on hepatocytes, endothelial or renal tubule cells might simply be a reflection of the cellular mechanisms for degrading and clearing the molecule.

The range of cell types available for *in vitro* testing of a candidate haemopoietic regulator is quite restricted and in many cases there are few parameters available for use in monitoring possible responses in such cells. It must be admitted therefore that tests on the validity of regarding the CSFs as exclusively haemopoietic regulators have not been exhaustive. Even so, there is some evidence to suggest that the CSFs may have some actions that are extrahaemopoietic. Thus M-CSF receptors are present on placental trophoblast cells and these cells can be stimulated to proliferate by M-CSF. The very high production rate of M-CSF in the pregnant uterus strongly suggests that M-CSF has an important role in placental function (Arceci *et al.* 1989). Similarly, injection of GM-CSF has been reported to reduce the frequency of spontaneous abortion in certain mouse strains (Wegmann *et al.* 1989), suggesting a possible influence also of GM-CSF on placental function.

One report has described the ability of both GM-CSF and G-CSF to influence the proliferation and migration of vascular endothelial cells (Bussolino *et al.* 1989). Although this awaits confirmation, it would not be a completely unreasonable possibility given the long-known involvement of endothelial cells in inflammatory responses as members of the old reticulo-endothelial cell system of Aschoff and Landau.

There have been reports that certain non-haemopoietic tumour cells can express CSF receptors and can be stimulated to proliferate *in vitro* of CSFs (Baldwin *et al.* 1989; Joraschkewitz *et al.* 1990). In some instances, this may be the consequence of abnormal gene expression in deranged cancer cells, but the possibility remains that the observations do have some bearing on normal physiology.

Contrary to these lines of evidence, however, are observations made on murine models in which sustained excess levels of one or other CSF have been generated, either by using transgenic animals or mice repopulated by CSF-producing haemopoietic cells. Pathological analysis of such animals has revealed only changes in haemopoietic populations and no proliferative or structural abnormalities in cells such as endothelial or lung cells.

For the present therefore, the CSFs do appear to be relatively restricted in their actions to haemopoietic cells, although the evidence is far from complete.

The quandaries raised by IL-6 and LIF are quite profound as it is evident that the body can choose to use the same molecule to regulate a quite broad range of cell types. There is no conceivable biological situation where the function of all of these cell types would need to be modulated simultaneously. What seems to be the most reasonable explanation is to propose that, for such molecules, the design system is that of local production and action rather than systemic production that might induce unwanted changes in other tissues.

To a degree, the CSFs show a similar design pattern. For example, GM-CSF is usually not present in the circulation and may well represent a molecule normally produced and acting only locally. In this latter situation, if GM-CSF did prove to have an action on some unrelated tissue it would still be able to function as a haemopoietic regulator if production of GM-CSF was usually restricted to sites of haemopoiesis.

These considerations do raise a certain unease about the validity of our current concepts about haemopoietic regulators and their role *in vivo*. It may well be that the present concepts of organ-specific biology and its

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regulation are either naive or incorrect. This would in no way detract from the clinical use of the CSFs as haemopoietic regulators, because they function in such situations as effective and essentially specific agents.

What does this work on haemopoietic regulators imply for the control of other tissue types? It seems reasonable to predict that the information concerning the haemopoietic regulator complex will be found to apply in principle to all other organ systems. If so, provided cell-culture techniques can be developed to monitor these cells, it seems probable that a wide range of comparable regulators will eventually be uncovered. These are exciting times for workers seeking to establish the mechanisms controlling the basic biological behaviour of mammalian cells and potentially exciting times for the clinician when such regulators become available for clinical exploitation.

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Lecture delivered 29 April 1991; typescript received 25 March 1991

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