

Cellular Specificity and Molecular Characteristics of the Binding of Colony-Stimulating Factors to Normal and Leukemic Cells

N. A. Nicola¹

A. Introduction

The colony-stimulating factors (CSFs) form a family of hemopoietic growth factors controlling the survival, proliferation and differentiation of hemopoietic progenitor cells as well as the functional activities of the mature cells in the tissues [1]. In the mouse, four distinct CSFs controlling granulocyte and macrophage production have been identified, purified and, in two cases, molecularly cloned – multi-CSF or interleukin-3, GM-CSF, M-CSF or CSF-1, and G-CSF [2, 3]. Human equivalents of GM-CSF (CSF α , NIF-T or pluripoietin α), G-CSF (CSF β or pluripoietin) and M-CSF have also been

purified and recently cloned [2–6] although no convincing equivalent of murine multi-CSF has yet been identified (Table 1).

A great deal is now known about the biological activities of each of these CSFs in vitro and more recently in vivo [7, 8], and these activities raise some interesting questions. First, some CSFs have broad cellular specificities (multi-CSF and GM-CSF) while other CSFs have very restricted or single-lineage specificities (M-CSF and G-CSF) (Table 1). How is this overlap in cellular specificities mediated and how do the CSFs co-ordinate to control individual cell populations? Second, some of the CSFs show a concentration dependence in the types of colonies stimulated – for example, at low concentrations GM-CSF stimulates the formation of only macrophage colonies, but at higher concentrations it also stimulates granulocyte

¹ The Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital 3050, Victoria, Australia

Table 1. Murine and human colony-stimulating factors^a

Murine CSF	Other names	Cell source	Cellular specificity	Human equivalent
Multi-CSF	Interleukin-3, PSF HCGF, MCGF, BPA	T cells WEHI-3B	G, M, Eo, E, Meg, Blast	?
GM-CSF	MGI-1GM, CSF-2	T cells Endothelial cells Macrophages	G, M, Eo	CSF- α , pluri- poietin- α , NIF-T
M-CSF	MGI-1M, CSF-1	Fibroblasts	M > G	M-CSF, CSF-1
G-CSF	MGI-1G, DF	Macrophages	G > M	CSF- β , pluripoietin

G, Granulocyte; M, macrophage; Eo, eosinophil; Meg, megakaryocyte; Mast, mast cell; MGI, macrophage, granulocyte inducer; HCGF, hemopoietic cell growth factor; MCGF, mast cell growth factor; BPA, burst-promoting activity; PSF, P-cell-stimulating factor; DF, differentiation factor.

^a For a list of references see Metcalf [2].

colony formation [9], while the reverse situation holds for G-CSF [10]. What is the cellular and molecular basis for this altered cell specificity with CSF concentration? Third, for normal hemopoietic progenitor cells the action of CSF results in a tight coupling between cell proliferation and cell differentiation, yet in factor-dependent cell lines the actions of multi-CSF and GM-CSF are associated only with cell proliferation [11] and in myeloid leukemias cell proliferation and differentiation are uncoupled or loosely coupled [12]. In some types of murine myeloid leukemias, G-CSF (but not the other CSFs) is able to overcome this uncoupling and induce terminal differentiation [13]. How is the coupling between proliferation and differentiation in normal progenitor cells mediated by CSFs, how does uncoupling occur in cell lines and leukemias, and why does G-CSF have a special role among the CSFs in differentiation induction? It was to try and answer some of these questions that we undertook a systematic study of the cellular specificities of CSF-binding interactions and a molecular analysis of the cross-interactions of CSFs at the receptor level.

B. Molecular Characteristics of CSF Receptors and Their Binding Interactions

Using radioiodinated derivatives of purified or recombinant murine CSFs the molecular nature of all four CSF receptors has now been determined by chemical cross-linking. The M-CSF receptor is a mol.wt. 165 000 [14] single-chain glycoprotein closely related or identical to the *c-fms* proto-oncogene [15], and the G-CSF receptor is a single-chain protein of mol.wt. 150 000 [16]. The GM-CSF receptor is a smaller (mol.wt. 55 000) protein [17] and, while the multi-CSF receptor is also relatively small, experiments have revealed cross-linking to two non-covalently attached subunits with mol.wt. of 75 000 and 60 000 [16] (Fig. 1). There is no evidence for different forms of these receptors on different normal cell populations, continuous cell lines or leukemic cells.

Binding studies at 4 °C and 37 °C have revealed that for all four CSFs the apparent

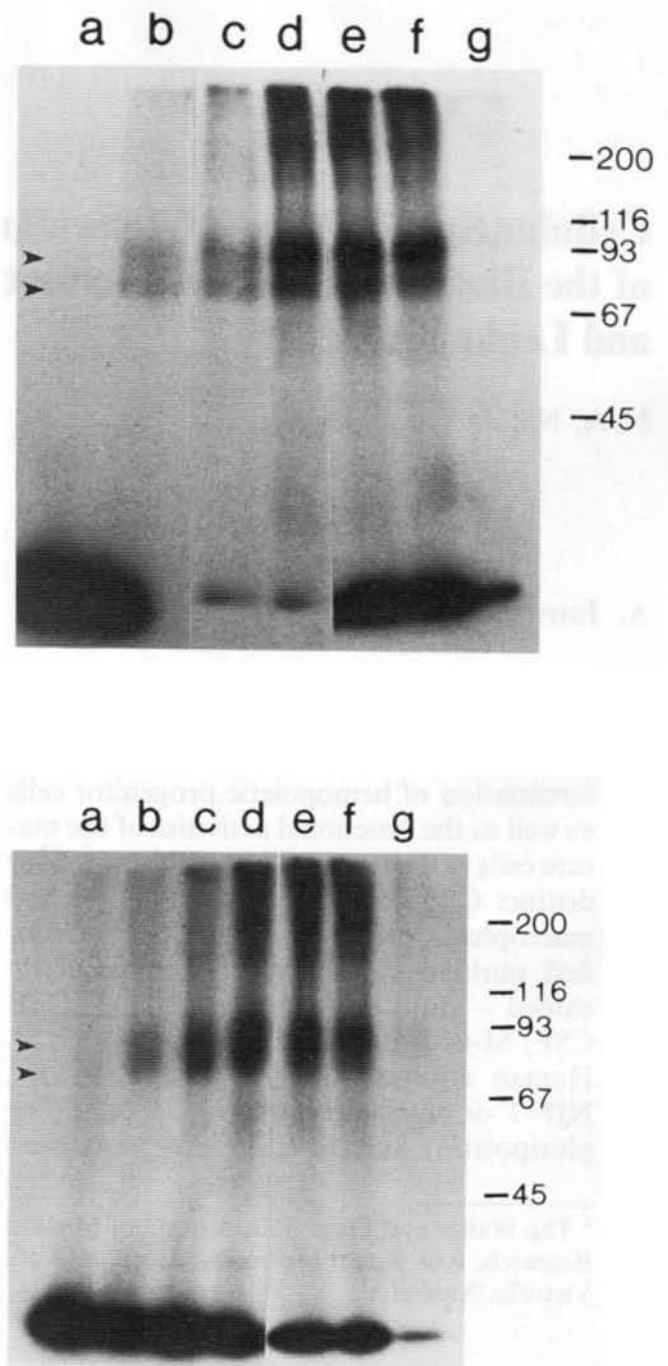


Fig. 1 Chemical cross-linking of ^{125}I multi-CSF to receptors on FDCP-1 cells. After binding of ^{125}I multi-CSF to FDCP-1 cells at 4 °C the cells were washed and incubated with increasing concentrations of the cross-linker disuccinimidyl suberate (DSS) at 4 °C (a-f, 0–10 mM DSS). Cell pellets were extracted with sodium dodecyl sulfate sample buffer, electrophoresed on 8% polyacrylamide gels in the presence (upper panel) or absence (lower panel) of dithiothreitol, and exposed for autoradiography. The position of molecular-weight markers (45–200 Kd) is shown on the right. After subtraction of the mol.wt. of cross-linked multi-CSF (15 Kd), the two proteins have mol.wts. of 75 000 and 60 000. Comparison of upper and lower panels indicates that these two proteins are not disulfide linked

dissociation constant is low (20 pM to 1 nM) and for most CSFs the number of receptors on responsive cells is low (less than a thousand receptors per cell) [17–22]. M-CSF is the exception, with some normal macrophages displaying up to 50 000 receptors per cell [23]. On normal cells or cell lines each receptor binding site appears to be unique and specific for its cognate ligand, with no cross-reactivity with other CSFs or several other growth factors tested when binding competition experiments are performed at temperatures of less than 10 °C. Based on the apparent dissociation constants for CSF binding and the known CSF concentrations required for half-maximal biological activity, it might be concluded that biological effects of the CSFs are exerted at low levels of receptor occupancy (i.e. there are spare receptors). This is especially surprising in view of the low levels of CSF receptors on responding cells, but this interpretation is complicated by the kinetic aspects of CSF binding interactions.

The kinetic rates of association of CSFs with their receptors are very fast, reflecting an essentially diffusion-controlled process. For G-CSF at 40 pM and 37 °C the half-time for association with receptors on murine leukemic WEHI-3B D⁺ cells is about 7 min and the kinetic association constant is $2.6 \times 10^9 \text{ M}^{-1} \text{ min}^{-1}$. In contrast, the kinetic dissociation rate is very slow ($T_{1/2}$ of 6 h at 0 °C and apparent $T_{1/2}$ of 5 h at 37 °C) even at high dilution of the cells with excess unlabeled G-CSF to prevent re-binding of ¹²⁵I G-CSF. Calculation of the dissociation constant from the ratio of kinetic dissociation to association constants gives a value of 1 pM for G-CSF binding to WEHI-3B D⁺ cells at 37 °C, a result much lower than that determined by Scatchard analysis of pseudo-equilibrium binding data (70 pM) but much closer to the concentration required for half-maximal biological effect (differentiation induction) on WEHI-3B D⁺ cells. This situation parallels that already described for M-CSF binding to macrophages [23, 24].

At 37 °C the apparent kinetic dissociation rate does not reflect only ligand dissociation from its receptor, and Scatchard analysis does not reflect a true equilibrium between ligand and receptor. This is because the

CSFs, like most other growth factors, are internalized along with their receptors after binding at 37 °C and are degraded intracellularly [24]. The binding data at 37 °C thus reflect a steady state (achieved within 1–6 h) where the rate of internalization and degradation of CSF-receptor complexes is balanced by the rate of expression of new receptors at the cell surface. The rates of internalization and degradation of CSF-receptor complexes vary for the different CSFs and for the same CSF in different cell types [24]. For example, M-CSF is rapidly internalized and degraded in macrophages ($T_{1/2}$ of several minutes), while multi-CSF is much more slowly internalized in bone marrow cells ($T_{1/2}$ of 1 h).

The slow dissociation kinetics of CSF-receptor complexes and their relatively rapid internalization at 37 °C mean that measurement of receptor levels on cells obtained from mice or on cell lines that need to be maintained continuously in CSF-containing media need to be interpreted with caution, since they will depend on the past history of the cells.

C. Cellular Specificity of CSF Binding

Several cell lines have been described that display receptors for one or more murine CSF. In principle, these cell lines should provide a homogeneous population of cells with which to study CSF-receptor interactions, but in practice autoradiographic analysis has revealed considerable intraclonal heterogeneity in receptor content not clearly related to cell cycle or differentiation status.

Of several murine cell lines tested, only two displayed specific binding of ¹²⁵I G-CSF – the J774 macrophage cell line and WEHI-3B D⁺ murine myelomonocytic leukemic cells which respond to G-CSF by differentiation induction. These two cell lines displayed very similar receptor numbers and apparent binding affinities for G-CSF [25]. Of special interest was the observation that the derived cell line WEHI-3B D⁻, which does not respond to G-CSF, had lost nearly all detectable binding sites for G-CSF, providing a ready explanation for its lack of inducibility for differentiation [18]. Cell au-

toradiographic analysis of the binding of ^{125}I G-CSF to murine bone marrow cells [19] revealed a cellular pattern of receptor distribution highly consistent with its biological specificity. Binding was restricted to neutrophilic granulocytes and their precursors and to a lesser extent to monocytic cells but was absent from eosinophils, erythroid cells and lymphocytes. Interestingly, receptor numbers were higher on neutrophils than on monocytes, were present on essentially all neutrophils and their precursors, and increased in number with cell differentiation [19]. The ability of G-CSF to stimulate functional activities of neutrophils is consistent with the presence of these receptors on post-mitotic cells [26].

G-CSF, unlike the other three murine CSFs, is fully cross-reactive on human cells, and this allowed a study of the distribution of human G-CSF receptors on human bone marrow cells and human leukemic samples [20]. On human cells binding was again restricted to neutrophilic granulocytes and their precursors and to a lesser extent, monocytic cells, but, in contrast to the situation in the mouse, receptor numbers decreased somewhat with neutrophil differentiation. This cellular specificity was maintained with human leukemias, in that all myeloid leukemias showed specific binding of ^{125}I G-CSF while lymphoid leukemias did not. The receptor content of myeloid leukemic cells was related to the granulocytic or monocytic nature of the leukemias, acute promyelocytic leukemias having the highest receptor content and monocytic leukemias having the lowest. Quantitative binding-competition studies revealed that murine and human G-CSF (CSF β) competed for the same binding sites on murine or human cells and that their relative binding affinities for all receptors were nearly the same when their respective concentrations were normalized with respect to a common bioassay [19].

M-CSF binds to a variety of monocytic cell lines (including WEHI-3B D $^+$ and J774) and to all normal populations of monocytic cells or macrophages but not to eosinophils, lymphocytes or erythroid cells [27]. There is some controversy about whether M-CSF binds to neutrophils and their precursors [27, 28], but if it does the receptor levels are

lower than on monocytic cells. There is some evidence that M-CSF receptors increase with differentiation of monocyte/macrophages [27, 28].

Multi-CSF binds to all factor-dependent continuous hemopoietic cell lines tested (FDCP-1, 32D Cl.3, DA-3, NSF-60) as well as some independent cell lines (P815 mastocytoma and WEHI-3B D $^+$) [21, 22], but the apparent binding affinities and receptor numbers vary widely. Multi-CSF also binds to all neutrophilic and eosinophilic granulocytes and all monocytes and their precursors but not to lymphocytes and erythroid cells [22]. In contrast to the binding patterns seen with G-CSF and M-CSF, there were similar multi-CSF receptor levels on neutrophils, monocytes and eosinophils, and in each case receptor numbers decreased with differentiation. ^{125}I multi-CSF binding to murine bone marrow cells was also characterized by the presence of a very small number (less than 1%) of cells of various morphologies (blasts, promonocytes, metamyelocytes, eosinophils) that displayed very high receptor levels similar to those seen on factor-dependent continuous cell lines [22].

GM-CSF also binds to several cell lines, including some factor-dependent cell lines (FDCP-1), some macrophage cell lines (J774, WR19, RAW 264, R309), and WEHI-3B D $^+$ myelomonocytic leukemic cells [17]. The distribution of receptors for GM-CSF on bone marrow cells is very similar to that of multi-CSF receptors, except that there are fewer GM-CSF receptors on eosinophils than on neutrophils or monocytes and there is no population of bone marrow cells with a very high GM-CSF receptor content (F. Walker, D. Metcalf, N. A. Nicola and A. W. Burgess, unpublished).

It is of special relevance that the majority of non-erythroid bone marrow cells display multiple CSF receptors. All neutrophilic granulocytes and their precursors display simultaneously receptors for multi-CSF, GM-CSF, G-CSF and possibly lower numbers of M-CSF receptors. All monocytic cells display receptors for multi-CSF, GM-CSF, and M-CSF, and a majority display lower numbers of G-CSF receptors. Eosinophils display multi-CSF and GM-CSF receptors but not G-CSF or M-CSF receptors. Lymphocytes and erythroid cells do not display

measurable levels of any of these CSF receptors. The display of multiple CSF receptors on granulocytes and macrophages means that they can respond to all of the CSFs directly and the possibility exists for receptor interactions on the same cell.

D. Indirect Receptor Modulations by the CSFs

Despite the fact that the four murine CSF receptors are distinct in both their molecular characteristics and their cellular distribution and that the binding of each CSF to bone marrow cells at 0 °C is not competed for by any of the other CSFs, there is evidence for indirect interactions between CSF receptors. When bone marrow cells were preincubated with one CSF at 37 °C the binding of other CSFs to their own receptors was reduced. This is an example of non-isologous receptor down-modulation, and it has been described for other growth factor receptor systems (for example, platelet-derived growth factor [29] and transforming growth factor β [30] both down-modulate epidermal growth factor receptors on some cell types).

The pattern of CSF-induced receptor down-modulations on bone marrow cells was quite striking, with multi-CSF being able to down-modulate all other CSF receptors, GM-CSF being able to down-modulate M-CSF and G-CSF receptors, and M-CSF being able to down-modulate GM-CSF receptors; G-CSF was able to down-modulate M-CSF receptors only at high concentrations. These down-modulations occurred at 37 °C but not at 0 °C, were generally rapid ($T_{1/2}$ 10–20 min) and were CSF-dose dependent [31] (Fig. 2). The common interpretation of receptor down-modulation is that it serves to limit the response of a cell to that receptor's growth factor. However, we were struck by several correlations between the ability of CSFs to down-modulate receptors and their ability to express certain biological activities that suggested an alternative interpretation.

First, CSFs with the ability to induce multiple types of differentiated cells had the broadest capacity to down-modulate CSF receptors (multi-CSF down-modulated all CSF receptors and GM-CSF down-modu-

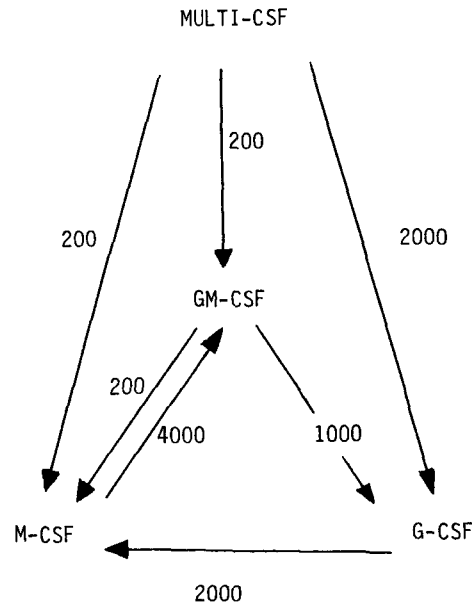


Fig. 2. Pattern and concentration dependence of CSF-induced receptor down-modulators on murine bone marrow cells at 37 °C. The arrows indicate the directionality of the ability of a particular CSF to down-modulate another CSF's receptor after pre-incubation of bone marrow cells at 37 °C. The numbers associated with each arrow are the concentrations of the CSFs (in bone marrow colony-stimulating units/ml) that result in 50% of the maximal down-modulation

lated G- and M-CSF receptors, while no CSFs other than multi-CSF down-modulated multi-CSF receptors). Second, the concentration dependence of CSF-induced differentiation matched the concentration dependence of that CSF's ability to down-modulate the appropriate CSF receptors (GM-CSF down-modulated M-CSF receptors at low concentrations and G-CSF receptors at high concentrations, G-CSF down-modulated M-CSF receptors at high concentrations). Third, certain agents that activate neutrophils also down-modulate G-CSF receptors on neutrophils (the chemotactic peptide *N*-formyl methionine leucine phenylalanine and bacterial lipopolysaccharide) [32].

These observations suggested that receptor down-modulation might result in activation of at least some of the biological activities of that receptor just as if it were occupied by the isologous ligand. Such a model proposes that multi-CSF and GM-CSF receptors deliver a mitogenic signal to a cell but, at the same time, indirectly activate lineage-

specific receptors that ultimately result in terminal differentiation. The advantages of such a model are: (a) it provides a unified explanation of the multiple differentiation specificities of some CSFs and their concentration dependence; (b) it provides a mechanism whereby proliferation and differentiation might be coupled in normal cells; (c) it makes clear predictions that such receptor cross-communication will be defective in some non-differentiating cell lines and leukemias and provides an explanation of the special role of G-CSF in differentiation induction of leukemias. With the availability of continuous cell lines and leukemic cell lines which display multiple CSF receptors, some of the predictions of this model can now be tested.

Acknowledgements. The original work reported here was supported by N.I.H. Grant CA-22556, the J. D. and L. Harris Trust Fund, the National Health and Medical Research Council of Australia and the Carden Fellowship Fund of the Anti-Cancer Council of Victoria. The technical assistance of Luba Oddo and Linda Peterson is gratefully acknowledged.

References

1. Metcalf D (1984) The hemopoietic colony-stimulating factors. Elsevier, Amsterdam
2. Metcalf D (1986) The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. *Blood* 67:257
3. Nicola NA, Vadas M (1984) Hemopoietic colony-stimulating factors. *Immunol Today* 5:76
4. Nomura M, Imazeki I, Oheda M, Kubota N, Tamura M, Ono M, Ueyama Y, Asano S (1986) Purification and characterization of human granulocyte colony-stimulating factor (G-CSF). *EMBO J* 5:871
5. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura M, Ono M (1986) Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. *Nature* 319:415
6. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chasin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsman R, Welte K (1986) Recombinant human granulocyte colony-stimulating factor: effects on normal and leukemic myeloid cells. *Science* 232:61
7. Kindler V, Thorens B, de Kossodo S, Allet B, Eliason JF, Thatcher D, Farber N, Vassali P (1986) Stimulation of hematopoiesis in vivo by recombinant bacterial murine interleukin-3. *Proc Natl Acad Sci USA* 83:1001
8. Metcalf D, Begley CG, Johnson GR, Nicola NA, Lopez AF, Williamson DJ (1986) Effects of purified bacterially synthesized murine multi-CSF (IL-3) on hemopoiesis in normal adult mice. *Blood* 68:46
9. Burgess AW, Metcalf D (1977) Colony-stimulating factor and the differentiation of granulocytes and macrophages. In: Baum SJ, Ledney GD (eds) *Experimental hematology today*. Springer-Verlag, Berlin Heidelberg New York, p 135
10. Metcalf D, Nicola NA (1983) Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. *J Cell Physiol* 116:198
11. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D (1980) Growth of factor-dependent hemopoietic precursor cell lines. *J Exp Med* 152:1036
12. Sachs L (1975) Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. *Nature* 274:535
13. Nicola NA, Metcalf D, Matsumoto M, Johnson GR (1983) Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells: identification as granulocyte colony-stimulating factor (G-CSF). *J Biol Chem* 258:9017
14. Morgan CJ, Stanley ER (1984) Chemical cross-linking of the mononuclear phagocyte-specific growth factor CSF to its receptor at the cell surface. *Biochem Biophys Res Commun* 119:35
15. Scherr CJ, Rettenmier CN, Sacca R, Roussel MF, Look AT, Stanley ER (1985) The *c-fms* proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. *Cell* 41:665
16. Nicola NA, Peterson L (1986) Identification of distinct receptors for two hemopoietic growth factors (granulocyte-colony stimulating factor and multipotential colony stimulating factor) by chemical cross-linking. *J Biol Chem* 261:12384
17. Walker F, Burgess AW (1985) Specific binding of radioiodinated granulocyte-macrophage colony-stimulating factor to hemopoietic cells. *EMBO J* 4:933
18. Nicola NA, Metcalf D (1984) Binding of the differentiation inducer, granulocyte colony-stimulating factor to differentiation responsive but not unresponsive leukemic cell lines. *Proc Natl Acad Sci USA* 81:3765

19. Nicola NA, Metcalf D (1985) Binding of ¹²⁵I-labeled granulocyte colony-stimulating factor to normal murine hemopoietic cells. *J Cell Physiol* 124:313
20. Nicola NA, Begley CG, Metcalf D (1985) Identification of the human analogue of a regulator that induces differentiation in murine leukaemic cells. *Nature* 314:625
21. Palaszynski EW, Ihle JN (1984) Evidence for specific receptors for interleukin-3 on lymphokine-dependent cell lines established from long-term bone marrow cultures. *J Immunol* 132:1872
22. Nicola NA, Metcalf D (1986) Binding of iodinated multipotential colony-stimulating factor (interleukin-3) to murine bone marrow cells. *J Cell Physiol* 128:180
23. Stanley ER, Guilbert LJ (1981) Methods for the purification, assay, characterization and target cell binding of a colony-stimulating factor (CSF-1). *J Immunol Methods* 42:253
24. Guilbert LJ, Stanley ER (1986) The interaction of ¹²⁵I colony-stimulating factor-1 with bone marrow-derived macrophages. *J Biol Chem* 261:4024
25. Nicola NA, Metcalf D (1984) Differentiation induction in leukemic cells by normal growth regulators: molecular and binding properties of purified granulocyte colony-stimulating factor. In: *Genes and Cancer*. Liss, New York, p 591
26. Lopez A, Nicola NA, Burgess AW, Metcalf D, Battye FL, Sewell WA, Vadas M (1983) Activation of granulocyte cytotoxic function by purified mouse colony-stimulating factors. *J Immunol* 131:2983
27. Byrne PV, Guilbert LJ, Stanley ER (1981) Distribution of cells bearing receptors for a colony-stimulating factor (CSF-1) in murine tissues. *J Cell Biol* 91:848
28. Shadduck RK, Pigoli G, Caramatti C, Degliontoni G, Rizzoli V, Porcellini A, Waheed A, Schiffer L (1983) Identification of hemopoietic cells responsive to colony-stimulating factor by autoradiography. *Blood* 62:1197
29. Bowen-Pope DF, D'Corleto PE, Ross R (1983) Interactions between the receptors for platelet-derived growth factor and epidermal growth factor. *J Cell Biol* 96:679
30. Assoian RK, Frolik CA, Roberts AB, Miller DM, Sporn MB (1984) Transforming growth factor- β controls receptor levels for epidermal growth factor in NRK fibroblasts. *Cell* 36:35
31. Walker F, Nicola NA, Metcalf D, Burgess AW (1985) Hierarchical down-modulation of hemopoietic growth factor receptors. *Cell* 43:269
32. Nicola NA, Vadas MA, Lopez AF (1986) Down-modulation of receptors for granulocyte colony-stimulating factor on human neutrophils by granulocyte-activating agents. *J Cell Physiol* 128:501