Activation and Proliferation Signals in Murine Macrophages: Synergistic Interactions Between the Hematopoietic Growth Factors and With Phorbol Ester for DNA Synthesis

By John A. Hamilton, Gino Vairo, Nicos A. Nicola, Antony Burgess, Donald Metcalf, and Suzanne R. Lingelbach

There has been recent interest in the synergistic interactions between the growth factors involved in the in vitro control of hematopoiesis and other cell lineages. As a convenient model system, such interactions governing the control of hematopoiesis and other cell lineages. As a convenient model system, such interactions involving the DNA synthesis in murine bone marrow-derived macrophages (BMMs) were studied. By themselves, murine colony-stimulating factor-1 (CSF-1) and recombinant murine granulocyte-macrophage CSF (GM-CSF) were stimulators of DNA synthesis in quiescent or noncycling BMMs, whereas recombinant murine interleukin-3 (IL-3) and the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), were weak mitogens. On the other hand, murine granulocyte CSF (G-CSF), concanavalin A (Con A), and lipopolysaccharide (LPS) were inactive on their own. When different growth factors (colony-stimulating factors, CSFs) acting on hematopoietic progenitor cells have now been purified and characterized.1 The generation of murine macrophages and granulocytes appears to be controlled by the interaction of at least four distinct CSFs.2 Unless used at high concentrations, CSF-1 (also called M-CSF)3 and granulocyte-CSF (G-CSF)4 show relatively restricted lineage specificity stimulating the production of macrophages or granulocytes, respectively. Granulocyte-macrophage CSF (GM-CSF) not only stimulates the production of mature granulocytes, macrophages, and eosinophils but can also initiate the proliferation of multipotent, erythroid, and megakaryocyte precursors.5 Multi-CSF,6 also called interleukin-3 (IL-3),7 stimulates the production of mature cells in most of the hematopoietic lineages, as well as the self-renewal of some multipotential stem cells.

Synergy between the different growth factors, which results in increased proliferation and/or differentiation of hematopoietic progenitor cells in vitro have been observed. For example, IL-3 synergizes with CSF-1-induced colony formation from primitive precursors and induces increased expression of CSF-1 receptors in populations of primitive marrow stem cells.6,7 Enhancing effects have also been observed between murine CSF-1 and a source of GM-CSF.8 Whether the four different CSFs recruit different populations of cells to proliferate or act on the same population to enhance total proliferative potential generally has not been addressed since purified cell populations and factors are required.

Evidence shows that the CSFs also act on the mature cells in the hematopoietic lineages, suggesting additional functions for these regulators (eg, refs, 9 through 11). We have been studying murine bone marrow-derived macrophages (BMMs) as a convenient and relatively homogeneous cell type to understand the biochemical responses of hematopoietic cells to CSFs.12,13 These cells require CSF-1 for their survival and proliferation and ≥95% contain high-affinity receptors for CSF-1,14 this receptor most likely being the tyrosine kinase product of the c-fms protooncogene.15 There is little information as to whether or not the CSFs have differential effects on the survival, proliferation, or function of macrophages. In this report, the effects of CSF-1, GM-CSF, IL-3, and G-CSF on BMM DNA synthesis are presented, as is evidence for some synergistic effects between the CSFs themselves and between the CSFs and the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). We also examined the effects of the macrophage-activating agents, concanavalin A (Con A) and lipopolysaccharide (LPS), on CSF-1-induced DNA synthesis and showed that LPS completely abolishes this response.

MATERIALS AND METHODS

Mice

Cells were from C3H/HeJ mice (6 to 12 weeks old, Walter and Eliza Hall Institute, Parkville, Australia) except in Fig 6 where cells from CBA mice were used.

BMMs

BMMs were obtained from precursor cells in bone marrow as described before.15 Femoral bone marrow cells were grown in 175-cm² (Lux) tissue culture flasks at 2 × 10⁶ cells/cm² for 3 days in 50 mL RPMI 1640 supplemented with 5 × 10⁻⁵ mol/L 2-mercaptoethanol, 20 mmol/L HEPES, 0.01% (wt/vol) neomycin.
sulfate, 15% heat-inactivated fetal bovine serum (FBS) and 20% L-cell conditioned medium (described below). For convenience and uniformity, the nonadherent population, containing primitive precursor cells, were collected, washed, and cryogenically preserved in liquid nitrogen in growth medium containing 10% dimethyl sulfoxide (DMSO) but without L-cell conditioned medium. For the DNA synthesis studies, these cells were thawed, washed, and seeded into 12-well dishes (Linbro; Miles Scientific, Naperville, IL) at 10^3 cells in 1 mL growth medium. Adherent BMMs were then grown to confluence for 5 to 6 days. The BMMs are a relatively pure and homogeneous population with >95% of the adherent cells binding CSF-1. At this stage, the cells were washed twice with sterile phosphate-buffered saline without Ca^2+ or Mg^2+, pH 7.4 (PD), and recultured in growth medium without L-cell conditioned medium. BMM were generally “starved” of growth factor for ~18 hours to produce the quiescent macrophages for the DNA synthesis studies. Such factor-deprived cells are in a quiescent G_0/G_1 state of the cell cycle. The protocol typically gave 3 x 10^5 adherent cells per well, measured by counting released nuclei as described previously.

**DNA Synthesis**

DNA synthesis was measured by incorporation of ^3H-thymidine (^3H-TdR) into trichloroacetic acid (TCA)-precipitable material. Quiescent macrophages (described above), were cultured in RPMI 1640/15% FBS with or without (control groups) a mitogenic stimulus for 20 hours at 37°C and then pulsed with ^3H-TdR (2.5 μCi/mL) for a further 2 hours. At this time, the medium was removed and the cells were washed four times with PD and solubilized in 0.2 mol/L NaOH. The solutions were made 10% in TCA, and the samples were filtered through a Millipore harvester using glass-fiber filters (Whatman GF/C; Maidstone, England). The filters were washed twice with 10% TCA, then twice with ice-cold H_2O and once with ethanol, and dried before β-scintillation counting.

**Sources of CSF-1**

BMM cultures. Serum-containing conditioned medium from mouse L6OT L-cells (L-cell CM) for BMM growth was prepared essentially as described.

**For experiments.** CSF-1 derived from serum-free L-cell CM was purified to homogeneity as described. In brief the purification stages were: concentrated serum-free L-cell CM (stage 1), calcium phosphate gel adsorption (stage 2), diethylamino ethanol (DEAE)-Sepharose chromatography (stage 3), Ultragel AcA-44 chromatography (stage 4), size-exclusion high-performance liquid chromatography (HPLC) (stage 5), reversed-phase HPLC (stage 6), and rechromatography on reversed-phase HPLC (stage 7). CSF-1 bioactivity was measured using C57Bl/6 bone marrow cells in semisolid agar medium, assigning 50 U/mL to the concentration giving half-maximal colony formation.

Another batch of CSF-1 used was also derived and purified from mouse placental conditioned medium by affinity chromatography using antibody. This was provided by T. R. Bradley and E. R. Stanley.

**IL-3 or Multipotent CSF (Multi-CSF)**

Native IL-3 was purified from medium conditioned by the clonal T-cell line LB-3 by sequential chromatography on red-A-Agarose (Amicon, Lexington, MA) (IL-3 was eluted at 0.3 mol/L KCl). Sephadex G-75 (Pharmacia, Uppsala, Sweden) (IL-3 was eluted with apparent mol wt 30,000), and reverse-phase HPLC on a phenyl-silica column (Waters’ fatty acid analysis column) (IL-3 eluted at 36% acetonitrile in 0.01% trifluoroacetic acid). This preparation gave a single silver-staining band on gel electrophoresis and had a specific activity of ~2 x 10^5 U/mg. (N. Nicola et al, unpublished observations).

The recombinant material (rIL-3) used was the nonglycosylated recombinant murine protein expressed in Escherichia coli and was provided in a partially purified form by J. Delamarter, Biogen SA, Geneva. This protein was further purified to homogeneity by reverse-phase HPLC on a Brownlee RP300 column and was free of detectable endotoxin (~0.06 ng/mL). Units of activity were obtained after assay on C57Bl/6 bone marrow cells, assigning 50 U/mL to the concentration giving half-maximal colony formation.

**GM-CSF**

The material used was bacterially synthesized material derived from E coli (rGM-CSF) and purified to homogeneity. The bioactivity was obtained after assay on C57Bl/6 bone marrow cells, assigning 50 U/mL to the concentration giving half-maximal colony formation.

**G-CSF**

Murine G-CSF was purified to homogeneity from lung-conditioned medium essentially as described; units of activity were determined and assigned for as the IL-3 and GM-CSF.

**Other reagents.** The following reagents were obtained commercially: [methyl-^3H] thymidine (70 to 85 Ci/mmol), TPA, 1-oleoyl-2-acetylglucerol (OAG), LPS (E coli 026: B6) (Cat. No. L1254), polyoxyn B sulfate (Sigma, St Louis), Con A (Miles-Yeda, Rehovot, Israel), RPMI 1640, Dulbecco’s modified Eagle’s medium (DMEM) and FBS (Commonwealth Serum Laboratories, Parkville, Australia), and BCA protein assay reagent (Pierce Chemical, Rockford, IL). All other reagents were of analytical grade. All practical precautions for minimizing endotoxin contamination were taken. Solutions were routinely made in pyrogen-free water (Abbott Hospital Products, Sydney, Australia), and endotoxin levels were routinely monitored by limulus lysate tests (Commonwealth Serum Laboratories, Parkville, Australia), with the minimal detectable levels being 0.1 ng/mL.

**RESULTS**

**CSFs and BMM DNA Synthesis**

In Fig 1, the DNA synthetic response to different CSFs are given. Figure 1A shows that recombinant GM-CSF (rGM-CSF) was a weak mitogen as compared to CSF-1. In the odd experiment, however, the relative stimulation indices for the two CSFs were quite similar, particularly if concentrations of GM-CSF were >10^4 U/mL (G. Vairo and J.A. Hamilton, unpublished observations); also the dose-response curves for purified native GM-CSF and rGM-CSF were similar (data not shown). In Fig 1A, 2,500 U/mL CSF-1 represented the maximal response to this growth factor, in agreement with the data in reference 16. As shown in Fig 1A, native IL-3 (nIL-3) was mitogenic but the effect was, as always, less than that for the CSF-1. At least for recombinant IL-3 (rIL-3), concentrations >10^4 U/mL were no more effective (Fig 4C). Figure 1B shows that G-CSF was not mitogenic at the concentrations used. If units based on the ability of CSFs to stimulate bone marrow colony formation at least are used, the data in Fig 1 indicate that the relative potencies of the CSFs as stimulators of BMM DNA synthesis are CSF-1 > GM-CSF > IL-3 > G-CSF (described in Discussion section).

The relative stimulatory activities of CSF-1, rGM-CSF, and nIL-3 were not altered if the ^3H-TdR was added at the same time as the CSFs for periods up to 3 days (data not...
shown), thus excluding a difference in the cycling times of BMMs in response to the different CSFs as an explanation.

**Synergism Between CSFs for BMM DNA Synthesis**

We then determined whether the other CSFs might synergize with CSF-1 to stimulate DNA synthesis. As shown for

---

**Fig 1.** Effect of CSF-1, IL-3, rGM-CSF, and G-CSF on BMM DNA synthesis. Quiescent BMMs (described in the Materials and Methods section) were treated with increasing concentrations of different CSFs in two experiments: (A) murine placental CSF-1, rGM-CSF, and nIL-3; and (B) L-cell CSF-1 (stage 3) and nG-CSF. Control groups contain simply RPMI 1640/15% FBS. DNA synthesis was measured as [3H]-TdR incorporation into TCA-precipitable counts (described in Materials and Methods section). Each point represents the mean of triplicate cultures (±SEM). Error bars are omitted when the errors were within the thickness of the border of the box.

**Fig 2.** Synergistic effects between CSF-1 and the other CSFs on BMM DNA synthesis. Quiescent BMMs (described in the Materials and Methods section) were treated with murine placental CSF-1 and different concentrations of the rGM-CSF or nIL-3 as follows: (A) rGM-CSF: no CSF-1 (○-○), 500 U/mL CSF-1 (●-●), 1,000 U/mL CSF-1 (X-X), 2,500 U/mL CSF-1 (■-■). (B) nIL-3: no CSF-1 (O-O), 500 U/mL CSF-1 (●-●), 1,000 U/mL CSF-1 (X-X), (■-■), 5,000 U/mL CSF-1. DNA synthesis was measured as [3H]-TdR incorporation into TCA-precipitable counts (described in the Materials and Methods section). Each point represents the mean of triplicate cultures (±SEM). Error bars are omitted when the errors were within the size of the symbol.
the experiment in Fig 2A, there was a dramatic effect between low concentrations of CSF-1 and rGM-CSF. At optimal concentrations of CSF-1 (≥2,500 U/mL), there was no evidence of any effect of the GM-CSF. The results with nGM-CSF again were similar to those with the recombinant form (data not shown). The same situation applied to low concentrations of CSF-1 and nIL-3 (Fig 2B). At the optimal concentration of CSF-1 (5,000 U/mL), the IL-3 again had no influence. Generally the degree of synergism between CSF-1 and GM-CSF was more pronounced than that between CSF-1 and IL-3. Synergistic effects with both GM-CSF and IL-3 were noted also when highly purified L-cell CSF-1 (stage 7 in the Materials and Methods section) was used (data not shown). For the combination of CSF-1 and G-CSF in four experiments, there was a weak, if any, synergistic effect (data not shown). IL-3 and rGM-CSF also were able to cooperate with each other to provide mitogenic signals for BMMs, although the degree of synergism was far less striking than with CSF-1 (Fig 3).

**TPA and BMM DNA Synthesis**

The phorbol ester, TPA, can synergize with CSF-1 in the development of macrophage colonies from bone marrow progenitors and is believed to act through protein kinase C. As shown in Fig 4A, TPA is a relatively weak mitogen as compared with CSF-1 but can synergize with suboptimal concentrations of CSF-1. At maximum CSF-1 doses (≥2,500 U/mL), TPA does not synergize with CSF-1 and, in several experiments it lowered the ³H-TdR incorporation by the CSF-1 (data not shown). We are unsure of the reason for this decrease, but it has been observed before with starch-induced murine peritoneal exudate macrophages.

The synergism by TPA extended to both rGM-CSF (Fig 4B) and rIL-3 (Fig 4C). The findings in the same experiment for the combined actions of CSF-1 and IL-3 are included for comparison.
concentrations of i-cell CSF-1 (stage 3) from left to right 0, 600, and 2,400 U/ml (O-O), and also with 50 μg/ml OAG (O—O), and 10^{-1} mol/L TPA (X—X). DNA synthesis measurement and data presentation as in Fig 2.

Fig 5. Synergistic effect between OAG and CSF-1 on BMM DNA synthesis. Quiescent BMMs were treated with different concentrations of L-cell CSF-1 (stage 3) from left to right 0, 300, 600, and 2,400 U/ml (O—O), and also with 50 μg/ml OAG (O—O), and 10^{-1} mol/L TPA (X—X). DNA synthesis measurement and data presentation as in Fig 2.

DNA synthesis measurement and data presentation as in Fig 2.

TPA is postulated to be acting as an analogue of diacylglycerol, the putative cellular activator of protein kinase C.26 As shown in Fig 5, the synthetic diacylglycerol, OAG, could also act synergistically with CSF-1 but its effect under our experimental conditions was much weaker than that with TPA. Further experiments are in progress to determine if there are more optimal conditions for OAG action.

Endotoxin, Con A, and BMM DNA Synthesis

We previously showed that both LPS and Con A activate BMMs, at least by the criteria of enhanced Na^+ ,K^+ -ATPase activity22 and glucose uptake.23 Because the CSFs and TPA also have these effects, LPS and Con A might be mitogenic or at least synergize with the CSFs. LPS (100 ng/ml) was not mitogenic for quiescent BMMs but inhibited quite dramatically the response to CSF-1 (Fig 6); there was no evidence that LPS-induced BMM toxicity could account for the negative result, as judged by cell morphology and cell counting. By mixing experiments, the inhibition by LPS could not be explained by the generation of an extracellular inhibitor (data not shown). Neither was Con A (10 μg/ml) mitogenic for the quiescent BMMs but, on the other hand, it had a very minor effect, if any, on the CSF-1 response. The interactions of LPS and Con A with the other CSFs have yet to be studied.

DISCUSSION

We have shown, using purified materials, that the four CSFs [CSF-1 (M-CSF), GM-CSF, IL-3 (multi-CSF), and G-CSF] differ markedly in their mitogenic activities for the mature cell type, BMM. Under the culture conditions we used, maximum DNA synthesis with CSF-1 occurs at ~2,500 U/ml (Fig 1A). Using published specific activities for purified CSF-1, this value is equivalent to 1.1 nmol/L 14 or 15 pmol/L.17 Maximal responses to rGM-CSF occurred in the range of 10^1 to 10^2 U/ml (ie, 0.15 to 1.5 nmol/L 21) although, for reasons unknown, the degree of responsiveness of the BMMs was usually less than that for CSF-1. Even though IL-3 had only weak stimulatory activity on quiescent BMMs, it can give rise to macrophage colonies from semisolid agar cultures of bone marrow cells1 as well as stimulate Na^+ ,K^+ -ATPase activity22 and glucose uptake23 in BMMs. Thus, macrophages retain some responsiveness to IL-3 even after differentiation (discussed in ref 28). G-CSF, on the other hand was essentially inactive with this lack of response being consistent with previous findings that G-CSF, unlike the other CSFs, fails to stimulate Na^+ ,K^+ -ATPase22 and glucose uptake23 in BMMs. Whether the fact that the BMMs used in this study were derived from progenitors by the action of CSF-1 had any influence on the pattern of activity described remains to be determined. It would be of interest to determine what would be the response patterns of macrophages that could be derived, for example, by the action of GM-CSF on progenitors instead of CSF-1.

When BMMs were exposed to combinations of the CSFs, there were striking synergistic effects on DNA synthesis for GM-CSF (Fig 2A) and IL-3 (Fig 2B) with suboptimal doses of CSF-1, with smaller effects for GM-CSF with IL-3 (Fig 3), and little if any effects for G-CSF with CSF-1 (data not shown). Such synergistic interactions between hematopoietic growth factors on immature (and more heterogeneous) cell populations have been described before. For example, enhancing effects between CSF-1 and a source of GM-CSF have been observed for murine bone marrow cells,2 and IL-3 (hematopoietin-2) synergizes with CSF-1-induced colony formation by murine bone marrow cells and induces increased numbers of CSF-1 receptors in populations containing primitive marrow stem cells.6 More recently, IL-3 was shown to enhance the proliferative capacity of both murine peritoneal exudate macrophages and monocytes exposed to CSF-1, apparently by increasing the binding of CSF-1 to the target cells.29

These biologic interactions among the CSFs could be explained either by processes such as receptor acquisition,6,29 hierarchical down-modulation (and activation) of
MACROPHAGE DNA SYNTHESIS: SYNERGISTIC INTERACTIONS 1579

other self-renewing tissues. Synergistic interactions between
ability to the synergistic actions between growth factors in
depth these mechanistic possibilities, which may have appli-
ties have been put forward for the CSFs.6'73' We propose
as competence or progression factors.3' All of these possibili-

ANCHORAGE-INDEPENDENT GROWTH OF THE SAME CELLS.33 The sys-
cytokine or macrophage formation, in Baum SJ, Ledney GD, Thierfelder
GM-CSF or M-CSF of bipotential GM progenitor cells to granulo-
Evidence for hierarchical difference in macrophage colony-
culture: [J Cell Physiol I 22:362,

 Some of these have already been observed for BMMs treated
such parameters as cytoplasmic alkalinization, cation and
from CSF-1 receptor activation
various pathways resulting from CSF-1 receptor activation
could coalesce at some stage(s), leading to increased DNA
synthesis. The synergistic effects with TPA (and OAG) point
to an interaction with protein kinase C. TPA treatment of
murine peritoneal exudate macrophages results in a rapid
loss of CSF-1 binding.39 This loss could be involved in the
increased transmission of proliferative signals; however, with
murine peritoneal exudate macrophages LPS has also been
observed to cause a loss of CSF-1 binding40 and to suppress
the DNA synthetic response of BMM to CSF-1 (Fig 6).

As mentioned, TPA, LPS, and Con A, as well as CSF-1,
GM-CSF, and IL-3, can activate BMMs by criteria such as
enhanced Na+/H+ antiport activity, Na+, K+-ATPase activity,
glucose uptake, etc.12,22,23 However, such macrophage
activation does not automatically lead ultimately to DNA
synthesis,22,23 so that there are crucial differences in the
responses of macrophages to activating agents, such as LPS
and Con A, and growth factors such as the CSFs.

ACKNOWLEDGMENTS

We thank S. Lingelbach for excellent technical assistance and
Mena Luciani for typing the manuscript. We also thank Drs E.R.
Stanley and T.R. Bradley for the sample of purified placental
CSF-1.

REFERENCES

Elsevier, New York, 1984
2. Burgess AW, Nicola NA: Growth Factors and Stem Cells.
Academic Press, Sydney, 1983
3. Stanley ER, Heard PM: Factors regulating macrophage
4. Nicola NA, Metcalf D, Matsumoto M, Johnson GR: Purifica-
tion of a factor inducing differentiation in murine myelomonocytic
leukemia cells: Identification as granulocyte colony-stimulating
5. Igle JN, Keller J, Henderson L, Klein F, Palaszynski EW:
Procedures for the purification of interleukin 3 to homogeneity. J
Immunol 129:2431, 1982
6. Bartelmez SH, Sacca R, Stanley ER: Lineage specific recep-
tors used to identify a growth factor for developmentally early
hemopoietic cells: assay of hemopoietin-2. J Cell Physiol 122:362,
1985
formation supported by purified CSF-1 and/or IL-3 in serum-free
culture: Evidence for hierarchical difference in macrophage colony-
8. Metcalf D, Merchav S, Wagemaker G: Commitment by
GM-CSF or M-CSF of bipotential GM progenitor cells to granulo-
cyte or macrophage formation, in Baum SJ, Ledney GD, Thierfelder
3
9. Hamilton JA, Stanley ER, Burgess A, Shadduck R: Stimula-
tion of macrophage plasminogen activator by colony stimulating
10. Hamilton JA: Control of macrophage stimulation and prolif-
11. Nicola NA, Vadas M: Hemopoietic colony-stimulating fac-
tors. Immunol Today 5:76, 1984
mediated 48Rb uptake in mouse bone marrow-derived macro-
glucose uptake in murine bone marrow-derived macrophages. Bio-
chem Biophys Res Commun 138:445, 1986
14. Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner
JR, Stanley ER: Survival of mononuclear phagocytes depends on a
lineage-specific growth factor that the differentiated cells selectively
15. Sherr CJ, Rettenmeir CW, Sacca R, Rousssel MF, Look AT,
Stanley ER: The c-fms proto-oncogene product is related to the
receptor for the mononuclear phagocytic growth factor, CSF-1. Cell
41:665, 1985
16. Tushinski RJ, Stanley ER: The regulation of mononuclear
phagocyte entry into S phase by the colony stimulating factor
17. Burgess AW, Metcalf D, Kozak J, Simpson RJ, Vairo G,
Hamilton JA, Nice EC: Purification of two forms of colony stimulat-
ing factor from mouse L-cell conditioned medium. J Biol Chem
260:16004, 1985
18. Stanley ER: The macrophage colony stimulating factor,
19. Kelso A, Glasebrook AL, Kanagawa O, Brunner KT: Produc-
tion of macrophage-activating factor by T lymphocyte clones and
correlation with other lymphokine activities. J Immunol 129:550,
1982
20. Kindler V, Thorens B, de Kossodo S, Allet B, Eliason JF,
Thatcher D, Farber N, Vassalli P: Stimulation of hematopoiesis in

www.bloodjournal.orgFrom www.bloodjournal.org by guest on October 21, 2017. For personal use only.
Activation and proliferation signals in murine macrophages: synergistic interactions between the hematopoietic growth factors and with phorbol ester for DNA synthesis

JA Hamilton, G Vairo, NA Nicola, A Burgess, D Metcalf and SR Lingelbach