Activation and Proliferation Signals in Murine Macrophages. Biochemical Signals Controlling the Regulation of Macrophage Urokinase-Type Plasminogen Activator Activity by Colony-Stimulating Factors and Other Agents

By John A. Hamilton, Gino Vairo, Kenneth R. Knight, and Benjamin G. Cocks

Purified hematopoietic growth factors such as colony-stimulating factor-1 (CSF-1) or macrophage colony-stimulating factor (CSF), and interleukin-3 or multi-CSF, stimulate the urokinase-type plasminogen activator (u-PA) activity of murine bone marrow-derived macrophages (BMM) and resident peritoneal macrophages. Granulocyte-CSF was inactive. The increases in BMM u-PA activity were inhibited by the glucocorticoid dexamethasone, and by agents that raise intracellular cyclic adenosine monophosphate levels, including prostaglandin E2 and cholera toxin. These changes in u-PA activity were paralleled by corresponding changes in u-PA mRNA levels. Evidence was obtained for protein kinase C and phospholipase C-mediated stimulation of BMM u-PA activity and mRNA levels; however, no evidence was found for an involvement of Na+/H+ exchange or Na+,K+-ATPase activity, Ca2+ fluxes, or pertussis toxin-sensitive G proteins. Several findings point to a dissociation between macrophage u-PA expression and DNA synthesis.

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A S FOR OTHER mature hematopoietic cells, macrophages derive from progenitor cells in the bone marrow through a coordinated process of proliferation and differentiation under the control of a group of glycoprotein hormones known as colony-stimulating factors or CSFs. The shortage of adequate numbers and/or sufficient purity of hematopoietic progenitor cells currently hinders attempts to determine the intracellular signals mediating CSF action. Cell lines are being used to study the mechanism of action of CSFs, including CSF-1 (or macrophage-CSF [M-CSF]), which acts on committed hematopoietic cells of the mononuclear phagocyte lineage. However, murine bone marrow-derived macrophages (BMM) are an easily obtainable homogeneous normal cell population useful for the study of the biochemistry of the action of CSFs. CSF-1 is responsible for the survival, proliferation, differentiation, and activation of macrophages and macrophage precursors at various stages of their development. As well as CSF-1, granulocyte-macrophage CSF (GM-CSF) and interleukin-3 (IL-3) (or multi-CSF) are also mitogenic for BMM, although not to the same degree as CSF-1. All of the CSFs synergize with each other in stimulating BMM DNA synthesis.

The actions of CSF-1 are mediated via binding to a specific surface receptor that has been shown to be the tyrosine kinase product of the c-fms proto-oncogene, and it is likely that the kinase activity is required for the cellular responses to CSF-1. The murine IL-3 receptor does not contain a consensus sequence for a tyrosine kinase in the cytoplasmic domain, although there is evidence for involvement of such a kinase in the signal transduction pathway. In murine hematopoietic cells the binding of GM-CSF to its receptor is not directly competed for by IL-3, although it can be indirectly down modulated at 37°C by IL-3. Despite the biochemical differences in the structures of these CSFs and their receptors, they all stimulate in BMM a variety of early responses that precede the onset of DNA synthesis, including stimulation of Na+/H+ exchange and/or Na+,K+-ATPase activity, an elevation of glucose uptake, c-fos, and c-myc mRNA levels. However, none of them activate the phosphatidylinositol-Ca2+-diacylglycerol cascade. The biochemical basis for these common actions is unknown.

Plasminogen activators (PAs) are serine proteases that cleave plasminogen to generate another serine protease, plasmin; there are two types of PA, urokinase-type PA (u-PA) and tissue-type PA (t-PA). A relationship between cellular proliferation and elevated u-PA mRNA levels has been proposed, being independent of the cell type and origin. BMM and murine peritoneal macrophages increase their PA activity in response to CSF-1, but the relationship to DNA synthesis is not completely clear. How CSF-1 regulates PA expression at the biochemical level is unknown.

PA activity has also been correlated with cell migration and tissue remodeling in a number of cellular systems, although there is little information on the signaling pathways involved in the control of PA synthesis by growth factors and hormones; in the particular case of the macrophage, PA activity has been correlated with inflammation and cell-mediated immunity. Uncharacterized lymphokine samples have been shown to elevate macrophage PA activity. The availability of purified recombinant cytokines has now made it possible to define which lymphokines or cytokines can activate macrophages by this criterion.

We report here on the effects of a number of purified recombinant murine CSFs on BMM u-PA mRNA expression and activity; this system is also used to analyze mechanisms and signaling pathways controlling both the increase and decrease in the expression of cellular u-PA activity. The relationship of u-PA activity to BMM DNA synthesis is also explored.
MATERIALS AND METHODS

Mice

Cells were obtained from male or female CBA or endotoxin-hyporesponsive C3H/HeJ mice (8 to 12 weeks old; Walter and Eliza Hall Institute, Parkville, Australia).

BMM

BMM were obtained from precursor cells in bone marrow as described before and grown to confluence for 5 to 6 days in RPMI-1640, 15% (vol/vol) fetal bovine serum (FBS), 20% (vol/vol) L-cell conditioned medium (CM) (see below). BMM were generally "starved" of growth factor for 18 to 20 hours before use to render cells in a quiescent G0/G1 phase of the cell cycle. BMM for PA assays were generally cultured in 24-well tissue culture dishes (Linbro; Flow Laboratories, McLean, VA) yielding ~1.5-2 x 10^6 adherent cells/well. Where indicated, some experiments were performed with BMM cultured in 12-well dishes (~3 to 4 x 10^6 adherent cells/well). For measurement of PA activity, the cells were washed twice with phosphate-buffered saline, pH 7.4, depleted of calcium and magnesium (PD), and resuspended in RPMI-1640/5% FBS from which plasminogen had been depleted (ATFBS-P). After the various additions, supernatants were collected at the indicated times and stored frozen until required for measurement of PA activity.

Resident Peritoneal Macrophages

Resident peritoneal cells were obtained from the peritoneal cavity as described and plated at ~1.5 x 10^6 cells per well in 1/2 fibrin-coated 96-well tissue culture plates (Linbro; Flow Laboratories, McLean, VA) yielding ~1.5-2 x 10^6 adherent cells/well. Where indicated, some experiments were performed with BMM cultured in 12-well dishes (~3 to 4 x 10^6 adherent cells/well). For measurement of PA activity, the cells were washed twice with phosphate-buffered saline, pH 7.4, depleted of calcium and magnesium (PD), and resuspended in RPMI/5% ATFBS with additions to a final volume of 200 µL.

PA Activity

PA activity released into the BMM culture medium was assayed essentially as described. Briefly, plasminogen-dependent fibrinolysis was measured as the release of soluble 125I-fibrin degradation products from 125I-fibrin-coated wells of 96-well plates. PA activity in the supernatants was expressed relative to the fibrinolytic activity of urokinase standards (0.06 to 2.0 Ploug U/well). Plasminogen-independent fibrinolysis was always ≤10% of plasminogen-dependent fibrinolytic activity.

For resident peritoneal macrophages cultured directly on 125I-fibrin-coated wells, PA activity was expressed as release of soluble 125I-fibrin degradation products directly into culture medium relative to the total radioactivity released by 0.25% (wt/vol) trypsin (ie, % fibrinolysis). The fibrinolytic activity from these cells has been shown to be plasminogen-dependent by deplasminogen, from the ATFBS used in the cultures during the assay period.

DNA Synthesis

DNA synthesis was measured as the incorporation of 3H-thymidine as described. Quiescent BMM, incubated in 12-well plates (Linbro) with the various treatments for 20 hours, were then pulsed with 3H-thymidine (2.5 µCi/mL) for a further 2 hours. Uptake was stopped, the cells solubilized in 0.2 mol/L NaOH, and the incorporation of label into trichloroacetic acid-precipitable material measured.

u-PA mRNA Expression

Isolation of total RNA. Quiescent BMM in 6-well plates (Costar, Cambridge, MA) were treated with the respective agents as indicated and the total RNA was isolated as described previously. Each sample represented the pool of the total RNA from three wells treated identically, 2 to 3 x 10^6 cells total.

Northern analysis. Northern analysis was performed as described previously. Samples of RNA were analyzed by formaldehyde agarose gel electrophoresis. RNA was transferred to Genescreen Plus (DuPont, Boston, MA) according to the manufacturer's instructions and hybridized to DNA probes radiolabeled by random priming. The murine urokinase probe was a 0.65-kb Polc-HindIII fragment of the cDNA plasmid pDB4501 kindly provided by D. Belin and J.D. Vassalli. The filters were washed and then exposed to Kodak XAR5 film (Eastman Kodak, Rochester, NY). The amount of RNA applied to each lane was monitored with a 32P-labeled cDNA to murine β2-microglobulin (the 0.3-kb Pol fragment from the murine β2-microglobulin gene).

Statistical Analysis of Data

Where indicated, statistical significance of measurements was determined by a two-tailed, unpaired Student's t-test. In all cases the results represent the average of triplicate determinations (±SEM).

Reagents

L-cell CM. For the establishment of BMM cultures, serum-containing CM from mouse L60T L cells (L-cell CM) was used as a source of CSF-1. This medium was essentially as described. Undiluted L-cell CM typically contained 8,000 to 12,000 U/mL CSF-1 (see below).

CSF-1. Purified recombinant human CSF-1 (rhCSF-1), which is biologically cross-reactive in the murine system, was used. This material was a gift of the Department of Process and Product Development, Cetus Corporation (Emeryville, CA), and the stock solution at 5 x 10^7 U/mL (see below) contained less than 0.015 ng/mL of endotoxin. In some experiments another source of CSF-1 was used that was derived and purified from mouse placental CM by affinity chromatography using antibody as described. This was kindly provided by T.R. Bradley and E.R. Stanley (Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, NY). Also, in some experiments, concentrated serum-free L-cell CM, subjected to calcium phosphate gel absorption (stage 2 L-cell CSF-1), was used as a source of CSF-1.

GM-CSF. Bacterially synthesized recombinant murine GM-CSF (rGM-CSF), purified to homogeneity, was provided by A. Burgess (Ludwig Institute for Cancer Research, Parkville, Australia).

IL-3. Two sources of murine IL-3 were used, being either recombinant or native material. The recombinant material (rIL-3) used was partially purified material produced by J. Delamarter (Biogen SA, Geneva, Switzerland) and further purified to homogeneity by N. Nicola (Walter and Eliza Hall Institute) as described before. The native material (nIL-3) derived from the clonal T-cell line LB-3 was purified to homogeneity as presented previously.

G-CSF. Murine G-CSF was purified to homogeneity from lung conditioned medium by N. Nicola.

CSF bioactivity. CSF 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.

Other reagents. The following reagents were obtained commercially: A23187, amiloride, 8-bromoadenosine 3',5'-cyclic monophosphate, concanavalin A (con A) (cat. no. C5275), dexamethasone,
fibrinogen, formyl-methyl-leucyl-phenyalanine (FMLP), 3-isobutyl-1-methyl-xanthine (IBMX), monensin, ouabain, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), phospholipase C purified from C-perfringens (cat. no. P4039), and 12-0-tetradecanoyl phospholipid-13-acetate (PMA) (Sigma, St Louis, MO); cholera toxin (CT) and ionomycin (Calbiochem, La Jolla, CA); lipopolysaccharide (LPS) from Escherichia coli 0111: B4, purified by the Westphal method (Difco, Detroit, MI); pertussis toxin (PT) (List, Campbell, CA); 1-oleoyl-2-acetylgllycerol (Serday, Ontario, Canada); RPMI-1640 and FBS (Commonwealth Serum Laboratories, Parkville, Australia); [methyld-<sup>3</sup>H] thymidine (70 to 85 Ci/mmol) and [α-<sup>32</sup>P] dATP (3,000 Ci/mmol) (Amersham, Australia). 5-N,N-dimethylamiloride was provided by E.J. Cragoe, Jr (Nagacloches, TX). All other reagents were of analytical grade. All practical precautions for minimizing endotoxin contamination were taken. Solutions were made in pyrogen-free water (Delta West, Bentley, Western Australia) and endotoxin levels were routinely monitored by limulus lysate tests (C.S.L., Parkville, Australia); the minimum detectable level being 0.1 ng/mL.

**RESULTS**

**Cytokine Stimulation of BMM PA Activity**

Various uncharacterized lymphokine preparations stimulate the PA activity of murine macrophages, but the identity(ies) of the stimulating activity(ies) has not been established.²²-²⁵ It has been found previously that highly purified L-cell-derived CSF-1 and murine GM-CSF from endotoxin-lung CM stimulated the PA activity of murine resident peritoneal macrophages and starch-elicited peritoneal macrophages.²⁶ Using BMM this time and with recombimant materials, we show that the lymphokines, GM-CSF, and IL-3 (Fig 1), but not G-CSF (Table 1), stimulate the PA activity. G-CSF showed no effect even if the cultures were continued for 72 hours (not shown). The data using purified CSF-1 are included for comparison. Thus, GM-CSF and/or IL-3 could have been contributing to the uncharacterized lymphokine activities mentioned above in prior studies with different macrophage populations.²²-²⁵

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<th>Additions</th>
<th>PA Activity (Ploug U/culture)</th>
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<tr>
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<td>11.5 (1.2)</td>
</tr>
<tr>
<td>CSF-1 (2,500 U/mL)</td>
<td>80.7 (8.6)</td>
</tr>
<tr>
<td>rG-CSF (10,000 U/mL)</td>
<td>14.3 (1.3)</td>
</tr>
<tr>
<td>rG-CSF (2,000 U/mL)</td>
<td>13.1 (0.5)</td>
</tr>
</tbody>
</table>

Quiescent C3H/HeJ BMM were treated with the indicated doses of G-CSF, CSF-1 (stage 2, L-cell CSF-1), or medium alone. PA activity released into the culture medium was measured after 24 hours as described in Materials and Methods. The values represent the mean of triplicate cultures (±SEM).

It has been shown previously that murine peritoneal macrophages express u-PA activity and mRNA in response to various stimuli.³²,³⁶ The molecular weight of the PA activity by sodium dodecyl sulfate-zymography in CSF-1-stimulated BMM culture supernatants was 48 Kd, a value obtained previously for murine macrophage u-PA.³⁷ The Northern analysis showed a transient induction of u-PA mRNA by CSF-1 with stimulation seen within 1 hour, reaching maximal levels between 4 and 8 hours with a decline to lower but still elevated levels at 24 hours (Fig 2A). Comparison of the CSF-1 dose responses for the stimulation of BMM PA activity (Fig 1) and u-PA mRNA induction (Fig 2B) shows a close correlation between both parameters. The RNA synthesis inhibitor, actinomycin D, inhibited the CSF-1-stimulated increase in PA activity (Table 2). Thus, CSF-1 would appear to be elevating u-PA activity in BMM by raising its mRNA levels possibly by increasing gene transcription. GM-CSF and IL-3 also increased u-PA mRNA levels in BMM, with these levels reflecting the degree of stimulation of PA activity by these agents (Fig 3). The phorbul ester, PMA, also dramatically increased u-PA mRNA levels in BMM. This is consistent with this agent also stimulating BMM PA activity (see below). The stimulation of BMM PA activity by GM-CSF, IL-3, and PMA was also inhibited by actinomycin D (data not shown), indicating that RNA synthesis is also required for stimulation of PA activity by these agents.

**Con A Stimulation of PA Activity**

The potent macrophage activating agent con A stimulated BMM PA activity. When measured at 24 hours in a representative experiment, 10 µg/mL con A increased the basal level of PA activity in CBA BMM from an average (±SEM) of 2.0 (0.4) to 21.5 (1.0) Ploug U/culture, which was comparable with the value of 23.5 (1.1) Ploug U/culture obtained in the presence of 2,500 U/mL rhCSF-1. Just as for the other agents, the stimulation of PA activity by con A was inhibited by actinomycin D (data not shown). Consistent with a requirement for RNA synthesis is the observation that con A also elevated the u-PA mRNA levels in BMM (Fig 3). However, at 3 hours the degree of stimulation of u-PA mRNA was somewhat lower than either GM-CSF (Fig 3) or CSF-1 (data not shown). We have found that the elevation of u-PA mRNA by con A is prolonged, showing greater relative stimulation at later

**Fig 1.** Stimulation of BMM PA activity by CSF-1, GM-CSF, and IL-3. Quiescent BMM, derived from C3H/HeJ mice, were treated with the indicated doses of murine placental CSF-1, rGM-CSF, or rIL-3. PA activity released into the culture medium was measured after 24 hours as described in Materials and Methods. Each point represents the mean of triplicate cultures (±SEM).
Table 2. Effect of Actinomycin D on CSF-1–Stimulated BMM PA Activity

<table>
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<th>Additions</th>
<th>PA Activity (Ploug U/culture)</th>
</tr>
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<tr>
<td>None</td>
<td>5.6 (0.5)</td>
</tr>
<tr>
<td>CSF-1 (2,500 U/mL)</td>
<td>53.2 (6.4)</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>1.3 (0.1)</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>8.0 (0.3)</td>
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</table>

Quiescent CBA BMM in 12-well dishes were pretreated with or without 2 μg/mL actinomycin D as indicated before the addition of recombinant murine CSF-1 or medium alone. PA activity released into the culture medium was measured after 8 hours as described in Materials and Methods. The values represent the mean of triplicate cultures (±SEM).

time points (ie, 24 hours) compared with the CSFs (data not shown). This is consistent with a previous report which showed that con A stimulation of u-PA mRNA levels in thioglycollate-elicited peritoneal macrophages requires 8 hours for detection. The positive actions of the cytokines and other agents are unlikely to be caused by endotoxin contamination because LPS (100 ng/mL) did not stimulate the PA activity in either the CBA- or C3H/HeJ-derived

Fig 2. Stimulation of BMM u-PA mRNA levels by CSF-1. (A) Kinetics: Northern analysis of u-PA and β₂ microglobulin mRNA in quiescent C3H/HeJ BMM. Total RNA was extracted (Materials and Methods) at the indicated times after the addition of 20% (vol/vol) L-cell CM as a source of CSF-1. The data are from the same Northern blot probed successively for u-PA and β₂ microglobulin mRNA. (B) CSF-1 dose response: Northern analysis of u-PA and β₂ microglobulin mRNA in quiescent C3H/HeJ BMM treated with the indicated doses of rhCSF-1 for 3 hours before the RNA extraction (Materials and Methods). The data are from the same Northern blot probed successively for u-PA and β₂ microglobulin mRNA.

Fig 3. Effects of GM-CSF, IL-3, PMA, and con A on BMM u-PA mRNA levels. Northern analysis of u-PA and β₂ microglobulin mRNA in quiescent CBA BMM treated with the appropriate agent for 3 hours before RNA extraction (Materials and Methods). The treatments are: lane 1, medium alone; lane 2, rGM-CSF (10,000 U/mL); lane 3, rIL-3 (10,000 U/mL); lane 4, PMA (10⁻⁷ mol/L); lane 5, con A (10 μg/mL). The data are from the same Northern blot probed successively for u-PA and β₂ microglobulin mRNA.
BMM (data not shown), or resident peritoneal macrophages (see Fig 8).

**Cyclic Adenosine Monophosphate (cAMP) and BMM PA Activity**

The biochemical pathways controlling the modulation of macrophage PA activity are not well understood. PGE₂ and CT (Fig 4A), which have been shown to raise intracellular cAMP levels ([cAMP]) in BMM, and in other cells, reduced both the basal and CSF-1–stimulated BMM PA activity. The results shown are for a 24-hour incubation, although the effect was observed as early as 3 hours. Similarly, the permeable cAMP analogue, 8-bromoadenosine 3’:5’-cyclic monophosphate (8BrcAMP) (1 mmol/L), and the cAMP phosphodiesterase inhibitor, IBMX (1 mmol/L), also completely abolished any detectable PA activity in the presence or absence of CSF-1 when measured at 7 to 8 hours (data not shown). The synthetic glucocorticoid, dexamethasone, has also been shown to inhibit u-PA induction in other cell types. In Fig 4A it can be seen that increasing concentrations of CSF-1 were not able to override the inhibitory effects of PGE₂ (10⁻⁹ mol/L), CT (10⁻¹⁰ mol/L), or of dexamethasone (10⁻⁷ mol/L) for stimulation of PA activity. In Fig 4B we show that PGE₂, 8BrcAMP, and CT inhibit the CSF-1–stimulated increase in u-PA mRNA levels. These agents alone have no effect on u-PA mRNA levels. Dexamethasone also inhibited CSF-1–stimulated u-PA mRNA levels (Fig 4C). Thus, modulation of u-PA mRNA induction is likely to be involved in the inhibition of CSF-1–stimulated BMM PA activity by cAMP and dexamethasone.

The elevation of [cAMP], has also been shown to corre-
late with inhibition of CSF-1-stimulated and also GM-CSF- and IL-3-stimulated BMM DNA synthesis. Fig 4D shows that the inhibitory effect of PGE₂, and to a lesser extent CT, were overcome at high CSF-1 concentrations. Similarly, inhibition by dexamethasone was also overcome at these CSF-1 doses. Thus, we can have experimental conditions where there is no PA expression yet DNA synthesis occurs, such as at high CSF-1 doses in the presence of PGE₂ or dexamethasone (Figs 4A and D). It has been suggested for other cell types that increased PA activity correlates with the subsequent expression of DNA synthesis.

In Table 3 it can be observed that CT (10⁻¹¹ mol/L) can also prevent the stimulation of BMM PA activity by the other active hematopoietic growth factors, GM-CSF and IL-3.

**G Proteins and BMM PA Activity**

Receptors for many hormones interact with membrane-bound guanine nucleotide-binding proteins (G-proteins), which can transduce signals leading to the generation of so-called second messengers. Certain G-proteins are sensitive to the effects of bacterial toxins such as PT. In Fig 5 it can be seen that 1 hour of pretreatment with PT (20 ng/mL) had no significant effect (P > .05) on the stimulation of BMM PA activity by the CSFs. Similar results were obtained with doses of PT up to 100 ng/mL and with pretreatment periods up to 5 hours (data not shown). This PT preparation was known to be biologically active because it ADP-ribosylated a 41-Kd protein in isolated rat islet cell membranes (M. Dunlop, personal communication). Similar results for BMM PA activity were obtained using several PT preparations obtained commercially. These PT preparations also had a pronounced morphologic effect on the BMM inducing a rounded, contracted morphology. The PT also had other effects on the cells including a partial inhibition of the basal levels of Na⁺, K⁺-ATPase activity, glucose uptake, and pinocytosis (data not shown).
Na⁺,K⁺-ATPase, Na⁺ Fluxes, and BMM PA Activity

Stimulation of Na⁺/H⁺ exchange by growth factors has often been considered to have an important role as an early signal in the proliferative response. We have evidence, including that based on the use of inhibitors, for the involvement of the Na⁺/H⁺ antiport and Na⁺,K⁺-ATPase activity (Na⁺ pump) in the proliferative response of BMM to CSF-1.42 However, we found that ouabain (1 mmol/L), a specific inhibitor of Na⁺,K⁺-ATPase activity,²⁹ had no significant effect on the induction of PA activity by CSF-1. In a representative experiment, 2,500 U/mL of rhCSF-1 increased the PA activity in C3H/HeJ BMM from an average (±SEM) of 1.2 (0.2) to 12.1 (0.4) Ploug U/culture during 8 hours of culture. In the presence of 1 mmol/L of ouabain these values were 1.2 (0.4) and 11.0 (0.7) Ploug U/culture, respectively. Unfortunately, we were not able to test the action of inhibitors of the Na⁺/H⁺ antiport activity, amiloride (1 mmol/L), and the more potent and specific analogue 5-N,N-dimethylamiloride (DMA) (0.1 mmol/L), because of their direct effects on u-PA proteolytic activity.²⁹ Therefore, we examined the effect of inhibitors of “Na⁺ cycle” ion transport on CSF-1–stimulated u-PA mRNA induction (Fig 6). Ouabain (1 mmol/L) did not inhibit CSF-1–stimulated u-PA mRNA induction. DMA (0.1 mmol/L) or Na⁺ depletion also did not inhibit the increase in u-PA mRNA levels stimulated by CSF-1. These results suggest that Na⁺,K⁺-ATPase activity and/or Na⁺/H⁺ exchange activity are not necessary for CSF-1–stimulated u-PA mRNA induction. Suppression of u-PA mRNA induction by amiloride (1 mmol/L) is unlikely to be related to Na⁺/H⁺ exchange inhibition because the more potent and specific inhibitor, DMA, did not have this effect. Amiloride is known to have a variety of nonspecific side effects and its inhibition of u-PA mRNA levels most likely reflects this. This result highlights the danger of relating the effects of amiloride to its action as an Na⁺/H⁺ exchange inhibitor.²⁹ We have published previously that CSF-1 rapidly stimulates BMM Na⁺/H⁺ antiport activity with the accompanying Na⁺ influx resulting in activation of the Na⁺,K⁺-ATPase activity (ie, CSF-1 stimulates an “Na⁺ cycle”).⁶² However, attempts to determine the importance of extracellular Na⁺ levels for stimulation of BMM PA by CSF-1 were hindered by the cytotoxic nature of prolonged exposure (4 hours) to Na⁺-depleted ([Na⁺] ≤ 5 mmol/L) conditions. Under these

Table 3. Effect of CT on CSF-Stimulated BMM PA Activity

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<th>Additions</th>
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<td></td>
<td>− CT</td>
</tr>
<tr>
<td>None</td>
<td>0 (1.0)</td>
</tr>
<tr>
<td>CSF-1 (5,000 U/mL)</td>
<td>45.5 (3.9)</td>
</tr>
<tr>
<td>rGM-CSF (10⁶ U/mL)</td>
<td>40.2 (4.2)</td>
</tr>
<tr>
<td>rIL-3 (10⁶ U/mL)</td>
<td>7.8 (0.4)</td>
</tr>
</tbody>
</table>

Quiescent C3H/HeJ BMM were pretreated with or without CT (10⁻¹¹ mol/L) as indicated for 1 hour before the addition of murine placental CSF-1, rGM-CSF, rIL-3, or medium alone. PA activity released into the culture medium was measured after 24 hours as described in Materials and Methods. The values represent the mean of triplicate cultures (±SEM).
conditions cellular protein synthesis was inhibited by ≥ 80%; however, there still remained a small degree of stimulation of PA activity by CSF-1 (approximately twofold) measured at 4 hours (data not shown). Under similar conditions there appeared to be little effect on CSF-1–stimulated u-PA mRNA levels (Fig 6). The Na⁺H⁺ exchange ionophore, monensin, results in cytoplasmic alkalinization as well as elevating intracellular [Na⁺] ([Na⁺]), which in turn activates Na⁺,K⁺-ATPase activity. Monensin was not able to increase PA activity, indicating that increased pH, elevation of [Na⁺], or stimulation of Na⁺,K⁺-ATPase activity alone was not sufficient to stimulate BMM PA activity (data not shown).

Protein Kinase C, Phospholipase C, Ca²⁺, and BMM PA Activity

Other possible biochemical pathways controlling BMM PA activity were explored. Diacylglycerols and the phorbol ester, PMA, are generally considered to activate protein kinase C, leading to a number of cellular effects. In Table 4 it can be seen that the synthetic diacylglycerol, 1-oleoyl-2-acetlylglycerol (OAG), and PMA were potent at increasing BMM PA activity. Stimulation of BMM PA activity by PMA is consistent with the result in Fig 3 that shows increased u-PA mRNA levels in response to PMA. 1,2-Diacylglycerols can arise intracellularly from phospholipase-mediated phospholipid hydrolysis. We show that treatment of BMM with highly purified, nonspecific phospholipase C stimulated BMM u-PA mRNA levels (Fig 7) and PA activity (Table 4).

An increase in [Ca²⁺], either by Ca²⁺ influx or release from intracellular compartments, has been implicated as having a second messenger role in a number of cell types. An increased Ca²⁺ influx by itself would not seem to be important for BMM PA production because the Ca²⁺ ionophores, A23187 and ionomycin, had no significant effect; the chemotactic peptide, FMLP, which can cause Ca²⁺ release from intracellular stores via activation of the phosphatidyl inositol cycle in BMM, was also inactive. In a representative experiment using C3H/HeJ BMM, a 24 hour treatment with A23187 (1 μmol/L), ionomycin (1 μmol/L), or FMLP (40 μmol/L) gave an average (±SEM) of 9.4 (2.4), 27.6 (4.5), and 17.9 (4.2) Ploug U/culture, respectively.

### Table 4. Effect of Protein Kinase C Activation and Phospholipase C Addition on BMM PA Activity

<table>
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<th>Additions</th>
<th>PA Activity (Ploug U/culture)</th>
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<td>CSF-1 (2,500 U/mL)</td>
<td>90.2 (7.3)</td>
</tr>
<tr>
<td>PMA (10⁻⁷ mol/L)</td>
<td>62.1 (6.1)</td>
</tr>
<tr>
<td>OAG (100 μg/mL)</td>
<td>77.9 (10.0)</td>
</tr>
<tr>
<td>PL-C (1 U/mL)</td>
<td>41.8 (1.4)</td>
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Quiescent C3H/HeJ BMM were treated with the indicated concentrations of murine placental CSF-1, TPA, OAG, PL-C, or medium alone. PA activity released into the culture medium was measured after 24 hours as described in Materials and Methods. The PL-C preparation had no detectable protease activity as measured by fibrinolysis. Each value represents the mean of triplicate cultures (±SEM).

These values were not significantly different (P > .05) to the basal PA activity of 15.2 (0.6). CSF-1 (2,500 U/ml; murine placental) increased the PA activity to 87.2 (1.5) Ploug U/culture in this experiment. These agents were also without effect on BMM u-PA mRNA induction (not shown).

Cytokine Stimulation of Resident Peritoneal Macrophage PA Activity

As mentioned, purified L-cell CSF-1 and murine GM-CSF from endotoxin-lung CM stimulated the PA activity of murine resident peritoneal macrophages when the cells were cultured on a fibrin substrate. We now show that purified rGM-CSF can enhance the PA activity of this type of macrophage (Fig 8). The effects of PMA, con A, and LPS are included for comparison. Purified rIL-3 (≥ 80 U/mL) was also stimulatory while purified G-CSF (≤ 1,000 U/mL)
Fig 8. Stimulation of resident peritoneal macrophage PA activity. Resident peritoneal cells were obtained from CBA mice and cultured on 3H-fibrin-coated 96-well plates as described in Materials and Methods. The cells were cultured overnight to allow adherence of the macrophages and the nonadherent cells were removed by two washes with PD. The adherent cells were cultured in RPMI/5% AThFBS, with or without the indicated doses of murine placental CSF-1, rGM-CSF, TPA (10⁻¹ mol/L), con A (10 μg/mL), LPS (100 ng/mL), or medium alone. Plasminogen-dependent fibrinolysis was measured as release of radioactivity into the medium after 24 hours and expressed relative to total radioactivity released by trypsin. Each point represents the mean of triplicate cultures (±SEM).

was not (data not shown). Thus, this poorly cycling macrophage population, as far as PA activity is concerned, can respond to the same agents as the BMM. With these cells, PA activity is not detected in the supernatants most likely because of the presence of PA inhibitor.

DISCUSSION

The above findings show that PA activity (ie, u-PA activity) joins a list of responses that the different CSFs (CSF-1, GM-CSF, and IL-3) are all able to elicit in BMM, and at similar doses, for the stimulation of other parameters, which include Na⁺/H⁺ exchange and/or Na⁺,K⁺-ATPase activity, glucose uptake, c-myc and c-fos proto-oncogene expression, pinocytosis (Knight KR, Vairo G, Hamilton JA: submitted for publication), and DNA synthesis. The lack of effect of G-CSF for BMM PA activity is consistent with its lack of effect on these other responses. This result most likely reflects the lack of functional G-CSF receptors for this cell type. The actions of CSF-1, GM-CSF, and IL-3, even though they are mediated via different receptors, can therefore converge on regulating u-PA activity and other BMM responses. The receptor for CSF-1 is the tyrosine kinase product of the c-fms proto-oncogene, while the receptors for GM-CSF and IL-3 do not appear to have tyrosine kinase activity. However, in FDC-P1 cells, IL-3 can induce the appearance of several phosphotyrosine-containing proteins, suggesting that a functional tyrosine kinase may be associated with this receptor.

Given the results above showing for the first time that rCSFs can increase u-PA mRNA levels (Fig 3) and given the inhibitory effects of actinomycin D (Table 2 and text), it is likely that the regulation of u-PA activity by the CSFs can occur at the level of gene expression.

Several possible signaling cascades were examined above to explore in more detail how the CSFs might be acting and how the u-PA activity might be regulated in macrophages. We and others have found previously no evidence for the phospholipase C-mediated hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PIP₂) in BMM treated with CSF-1, GM-CSF, or IL-3. Therefore, this pathway, leading to the formation of 1,2 diacylglycerol and inositol phosphates, would not seem to be involved in the action of these CSFs including, of course, the regulation of BMM PA activity. However, the stimulatory action of nonspecific phospholipase C (Table 4), which presumably can increase levels of intracellular diacylglycerol, suggests that this product(s) of phospholipid breakdown may be important to the modulation of u-PA activity and other responses in macrophages. Although treatment of BMM with nonspecific phospholipase C does not stimulate PIP₂ hydrolysis, such treatment has been found to stimulate BMM DNA synthesis and c-fos and c-myc expression. The stimulatory action of exogenous OAG (and PMA, see below) for macrophage PA activity also supports this proposal. It could be that the CSFs studied here elevate the levels of diacylglycerol in macrophages (as has recently been found in human monocytes) and this possibility is currently being explored. It is also possible that intracellular diacylglycerol may be involved in the control of u-PA activity in other cell types.

Diacylglycerol is the endogenous second messenger associated with activation of protein kinase C. The phorbol ester is a relatively weak mitogen for BMM but can synergize quite dramatically with a low concentration of CSF-1, GM-CSF, and IL-3 to potentiate their mitogenic action. The results reported here show that PA activity is another response in BMM that PMA shares with the different CSFs, the other responses including enhanced "Na⁺ cycle" activity, glucose uptake, proto-oncogene expression, pinocytosis (Knight KR, Vairo G, Hamilton...
The data above suggest that CSF-mediated stimulation of BMM u-PA mRNA levels and/or activity does not involve PT-sensitive G proteins (Fig 5) nor activation of Na⁺/H⁺ exchange or Na⁺,K⁺ ATPase activity (Fig 6) to any significant extent. In contrast, PT-sensitive G proteins have been implicated in the modulation of human monocyte Na⁺,K⁺-ATPase activity and DNA synthesis by CSF-1,⁶³ although in murine BMM, PT had only a minor effect on CSF-1-stimulated cell growth.⁶⁴ Also, Na⁺ and Ca²⁺ influxes by themselves do not seem to be capable of stimulating BMM u-PA activity (see Results). Ca²⁺ has been proposed as an important mediator of macrophage activation.⁶⁵

The inhibition of CSF-stimulated u-PA mRNA levels and activity in BMM by agents that raise [cAMP] in BMM⁶⁶ and other cell types,⁶⁷ such as 8BrCAMP, CT, PGE₂, and IBMX, suggests that elevation of [cAMP] is inhibitory to CSF-stimulated u-PA activity. We have found previously, when measuring the inhibition by these agents of CSF-stimulated DNA synthesis in BMM, that the effect was reversible, nontoxic, and without any effect on protein synthesis or other early CSF-1 responses.⁶⁸ u-PA activity is the one early response in BMM to the CSFs so observed thus far that is inhibited by the cAMP-elevating agents.⁷ The inhibition by agents that raise [cAMP] would not appear to result from interference with the binding of at least CSF-1, GM-CSF, IL-3, and PMA with their receptors because we have reported that other biochemical changes induced by these agents, such as c-myc expression, Na⁺/H⁺ exchange, Na⁺,K⁺-ATPase, and protein synthesis, were not affected by them⁶⁷ (Vairo G, Hamilton JA: unpublished observations); in addition, PGE₂ has been reported not to inhibit binding of CSF-1 to its receptor on BMM.⁶⁸ Agents that elevate cAMP, and also dexamethasone, have been shown to lower the PA activity and the basal transcription rate of u-PA gene expression in murine thioglycollate-elicited peritoneal macrophages,⁶⁹ given the reduction in the mRNA levels, it is likely that the inhibitory effects on u-PA activity are due to such a mechanism in the CSF-stimulated BMM.

It has been proposed that increases in PA activity in response to mitogenic agents may be important for the subsequent onset of DNA synthesis in CSF-1-treated BMM⁷⁰ and in other cell types.⁷¹ The stimulation reported above of the u-PA activity of BMM by the mitogenic agents, CSF-1, GM-CSF, IL-3, PMA, and phospholipase C would appear to support this concept, as would the observation that cAMP-elevating agents and dexamethasone inhibit mitogen-induced u-PA activity and can also inhibit DNA synthesis in BMM. However, there are several observations with macrophages that provide evidence against a relationship between stimulation of PA activity and DNA synthesis: (1) at high CSF-1 doses, PGE₂ and dexamethasone completely abolish CSF-1-stimulated BMM PA activity with no significant effect on CSF-1-stimulated DNA synthesis (Fig 4A and D); (2) DMA and ouabain both inhibit CSF-1-stimulated BMM DNA synthesis⁶⁸ but have little effect on CSF-1-stimulated u-PA mRNA levels and/or u-PA activity (Fig 6); (3) con A stimulates u-PA activity in BMM but is not mitogenic;⁶⁸ (4) CSF-1 stimulates the PA activity of murine resident peritoneal macrophages (Fig 8), with this cell type showing a poor proliferative response to the growth factor;⁶⁹ (5) untreated thioglycollate-elicited murine peritoneal macrophages express high levels of u-PA activity (~52% of the cells express PA activity) yet do not cycle unless treated with a growth stimulus;⁶⁴ (6) for CSF-1-treated BMM, the induction of PA activity is not a signal for DNA synthesis because an additional macromolecular serum component is required for CSF-1-stimulated DNA synthesis but not PA activity.¹⁷

Interestingly, con A, which potently stimulates BMM u-PA activity, has no effect on c-fos or c-myc mRNA levels in these cells.⁷² This result indicates that, for at least con A as a stimulus, expression of these proto-oncogenes is not a prerequisite for that of u-PA.

Elevated macrophage PA activity has been correlated with the degree of cell-mediated immunity in murine models and uncharacterized lymphokine-containing samples have been found to stimulate the PA activity of murine peritoneal macrophage populations.²²-²⁵ Interferon-γ has been found to increase u-PA gene transcription in murine peritoneal exudate macrophages.³¹ The fact that purified recombinant GM-CSF and IL-3 could enhance the PA activities of BMM and resident peritoneal macrophages suggests that one or both of these cytokines could have been responsible for the previous findings with the ill-defined lymphokine preparations mentioned.²²,²³ It is also possible that CSF-1 may have contributed to the stimulatory activities in these previous reports because this CSF can be produced by monocytes and unfractionated mononuclear cells were usually used for the preparation of the lymphokine-containing samples. Because PA has been implicated in inflammatory reactions, tissue remodeling, and cell migration,¹⁴,¹⁵,²⁵ GM-CSF and IL-3 (as well as CSF-1) may therefore have a role in these processes via their action on macrophages. Such enhanced u-PA production (and therefore plasmin generation) by the macrophages in GM-CSF transgenic mice may contribute to the necrotic lesions of the eye and striated muscle, as well as to the chronic bleeding associated with the unusually large numbers of peritoneal macrophages.³⁸ These points have been made elsewhere where it was reported that GM-CSF and IL-3 can stimulate in an analogous manner the u-PA activity of human monocytes;⁷³,⁷⁴ thus our observations have a parallel with human cells.

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