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GM-CSF AND IL-3 STIMULATE DIACYLGLYCEROL GENERATION IN MURINE BONE MARROW-DERIVED MACROPHAGES

Nurin Veis* and John A. Hamilton

University of Melbourne, Department of Medicine, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia

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SUMMARY In murine bone marrow-derived macrophages, prelabeled with either [^{3}H]myristic acid or [^{3}H]arachidonic acid, the mitogenic colony stimulating factors GM-CSF and IL-3 stimulated a transient increase in [^{3}H]diacylglycerol generation. Maximum [^{3}H]diacylglycerol levels were detected at 10-15 min. The stimulation of [^{3}H]diacylglycerol generation was dependent on the concentration of CSF and correlated with their ability to activate a variety of processes in the macrophage, including DNA synthesis. This is the first report to demonstrate that GM-CSF elevates diacylglycerol levels in macrophages and also to show that diacylglycerol generation may be an important signaling mechanism for IL-3 action. In conjunction with our recent demonstration that the mitogenic agents CSF-1, 12-0-tetradecanoylphorbol-13-acetate and exogenous phospholipase C also stimulate diacylglycerol generation in the macrophage (Veis and Hamilton, *J.Cell.Physiol.*, 147, 298-305, 1991), our findings suggest that an increase in diacylglycerol levels is necessary but not sufficient for macrophage proliferation.

Macrophage proliferation is under the control of the colony stimulating factors CSF-1, GM-CSF and IL-3 (1,2). In murine bone marrow-derived macrophages, these colony stimulating factors stimulate a variety of early events that precede the onset of DNA synthesis, including stimulation of Na^+/H^+ exchange (3), Na^+, K^+-ATPase activity (4), glucose uptake (5), c-fos and c-myc mRNA levels (6), pinocytosis (7,8) and plasminogen activator production (9). Inositol phosphate levels are not elevated in macrophages by CSF-1, GM-CSF and IL-3 (10,6), however CSF-1 does stimulate DAG generation in murine BMM (11) and human monocytes (12). We have investigated the effects of GM-CSF and IL-3 on the generation of

*To whom correspondence should be addressed.

ABBREVIATIONS

A-DAG, [^{3}H]arachidonyl-DAG; BMM, bone marrow-derived macrophage; CSF, colony stimulating factor; DAG, diacylglycerol;FBS, fetal bovine serum;GM-CSF, granulocyte, macrophage-colony stimulating factor; IL-3, interleukin-3; L-CM, L-cell conditioned medium; M-DAG, [^{3}H]myristoyl-DAG; PKC, protein kinase C; PLC, phospholipase C; SEM, standard error of the mean; TPA, 12-0-tetradecanoylphorbol-13-acetate.

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both [^{3}H]myristoyl-DAG (M-DAG) and [^{3}H]arachidonyl-DAG (A-DAG) in BMM. We show for the first time in this report that GM-CSF and IL-3 elevate DAG generation in macrophages. In conjunction with the findings in our recent report (11) it would appear that all agents that stimulate DNA synthesis in BMM elevate both M-DAG and A-DAG levels in these cells. However, the stimulation of DAG generation appears to be necessary but not sufficient on its own for macrophage proliferation.

MATERIALS AND METHODS

Mice

Cells from CBA and endotoxin-hyporesponsive C3H/HeJ mice (6-10 week old, Walter and Eliza Hall Institute, Parkville, Australia) were used in this study.

Bone marrow-derived macrophages

BMM were cultured from precursor cells obtained from bone marrow as described (13) and grown to confluence for 5-6 days in RPMI 1640 medium supplemented with $5x10^{-5}$M 2-mercaptoethanol, 20mM HEPES, 0.1 g/liter neomycin sulfate, 15% (v/v) heat-inactivated fetal bovine serum (FBS), and 20% (v/v) L-cell conditioned medium (CM), which is a crude source of CSF-1 (see below). BMM were "starved" of CSF-1 for 18-20h prior to use. Such factor-deprived cells are in a quiescent G_o/G_1 phase of the cell cycle (15).

Extraction and analysis of lipids

Diacylglycerol generation was estimated by incubating BMM (~10^6 cells) in 6-well plates for 20h in 1.0 ml RPMI 1640 containing 10% (v/v) FBS and 4 μCi/ml of either [^{3}H]myristic acid or [^{3}H]arachidonic acid and levels of [^{3}H]diacylglycerol were measured as previously described (11). Data were normalized to the non-stimulated control value.

Reagents

L-cell conditioned medium BMM cultures were supplemented with serum-containing conditioned medium from mouse L60T L cells (L-cell-CM) which was used as a crude source of CSF-1 (13).

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

IL-3 Recombinant murine IL-3, purified to homogeneity, was provided by Biotechnology Australia Pty Ltd., Roseville, Australia.

CSF bioactivity CSF bioactivity was measured using the C57Bl/6 bone marrow cells in semi solid agar medium assigning 50 U/ml to the concentration giving half-maximal colony formation (17).

Other reagents [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (7.73 TBq/mmol), [9,10(n)-^{3}H]myristic acid (1.98TBq/mmol) and Amplify Spray were purchased from Amersham. Neutral lipid standards were supplied by Sigma (St. Louis, MO). Tissue culture media and supplies were obtained from Commonwealth Serum Laboratories (Parkville, Australia). Other reagents were of analytical grade. All practical precautions for minimising endotoxin contamination were taken. Solutions were routinely made in pyrogen-free water, and endotoxin levels were regularly monitored by limulus lysate tests (Commonwealth Serum Laboratories, Australia), with minimum detectable levels being 0.01 ng/ml.

Statistics Statistical comparisons were performed using Student's two-tailed t test for unpaired samples, significance being taken at the 5% level ($p<0.05$).

RESULTS

Effect of GM-CSF and IL-3 on DAG generation

Kinetics Both [^{3}H]myristoyl-DAG (M-DAG, Figure 1) and [^{3}H]arachidonyl-DAG (A-DAG, Figure 1 insert) levels were maximally elevated within 10-15 min of exposure to GM-CSF (at

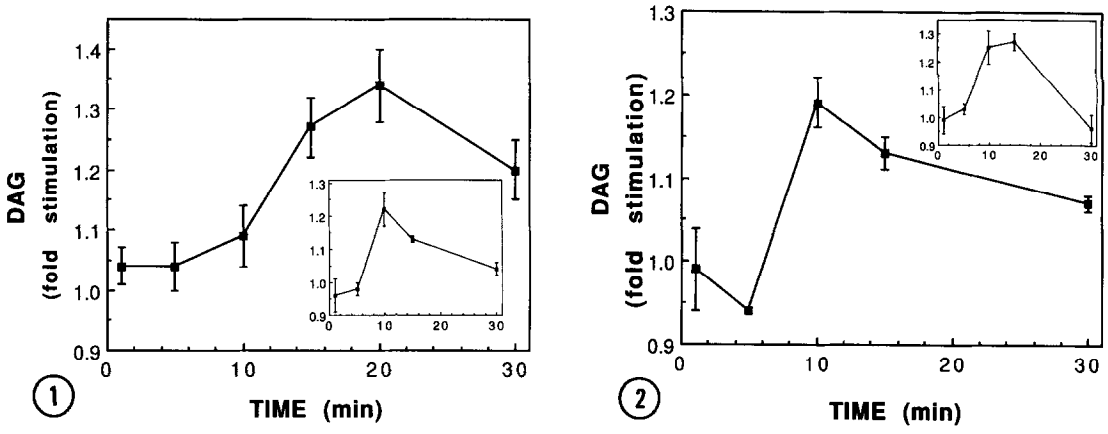


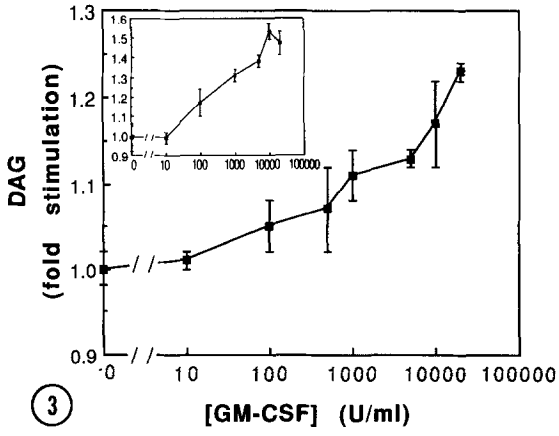
Figure 1. **Kinetics of DAG generation in BMM in response to GM-CSF.**
The effect of 10^4 U/ml GM-CSF on [^{3}H]myristoyl-DAG and [^{3}H]arachidonyl-DAG (inset) generation in quiescent BMM. Labeled cells were exposed to GM-CSF for the indicated times and the levels of [^{3}H]DAG determined as described in Methods. Data expressed as mean ± SEM values for triplicate determinations in a representative experiment. Similar observations were made in two other experiments. The total amount of labeled DAG measured in untreated cells at 15 min was 13,200 ± 390 dpm (M-DAG) and 21,870 ± 150 dpm (A-DAG).

Figure 2. **Kinetics of DAG generation in response to IL-3.**
The effect of 10^4 U/ml IL-3 on [^{3}H]myristoyl-DAG and [^{3}H]arachidonyl-DAG (inset) generation in quiescent BMM. Labeled cells were exposed to IL-3 for the indicated times and the levels of [^{3}H]DAG determined as described in Methods. Data expressed as mean ± SEM values for triplicate determinations in a representative experiment. Similar observations were made in two other experiments. The total amount of labeled DAG measured in untreated cells at 15 min was 4,320 ± 60 dpm (M-DAG) and 21,870 ± 150 dpm (A-DAG).

15 min $p<0.01$ (M-DAG), at 10 min $p<0.01$ (A-DAG)). The elevation of [^{3}H]DAG levels was transient for both forms and values restored to basal within 30-60 min. This pattern and magnitude of M-DAG and A-DAG generation are similar to those observed following exposure to CSF-1 in BMM (11).

IL-3 also stimulated a transient increase in both M-DAG (Figure 2) and A-DAG (Figure 2 inset) levels in labeled BMM. A maximal elevation in DAG was detected at 10 min ($p<0.01$, M-DAG and A-DAG) with levels gradually returning to basal values within 30-60 min. In more than half of the experiments performed a very transient 10-15% increase in both M-DAG and A-DAG was detected following 30 sec exposure to either GM-CSF or IL-3, but this disappeared by 1 min (data not shown). To date, there does not appear to be a significant increase in DAG at times earlier than 30 secs as measured by this isotopic method. Other experiments are currently underway to explore this early spike of DAG generation in response to GM-CSF and IL-3 in more detail.

Dose response GM-CSF stimulated the increase in BMM M-DAG in a dose-dependent manner (Figure 3). This was also observed for A-DAG (Figure 3 inset). Significant



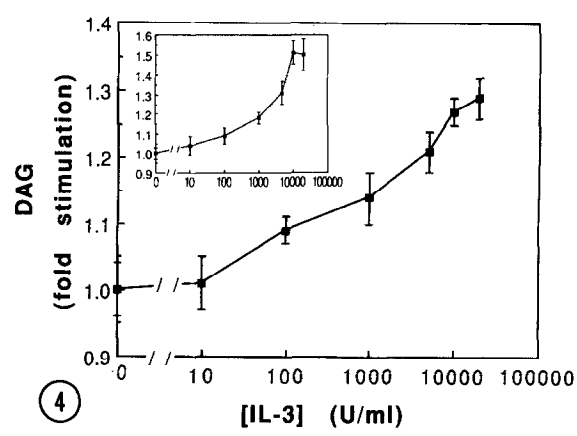


Figure 3. **GM-CSF dose response.**
GM-CSF-mediated stimulation of [^{3}H]myristoyl-DAG and [^{3}H]arachidonyl-DAG (inset) levels in BMM. Quiescent labeled BMM were exposed to varying concentrations of GM-CSF for 15 min at 37°C. Levels of [^{3}H]DAG were determined as described in Methods. Data shown as mean ± SEM values for triplicate determinations in a representative experiment. Similar observations were made in two other experiments. Total amount of labeled DAG measured in untreated cells were 11,080 ± 20 dpm (M-DAG), and 12,830 ± 770 dpm (A-DAG).

Figure 4. **IL-3 dose reponse.**
IL-3-mediated stimulation of [^{3}H]myristoyl-DAG and [^{3}H]arachidonyl-DAG (inset) levels in quiescent BMM. Labeled cells were exposed to varying concentrations of IL-3 for 15 min at 37°C. Levels of [^{3}H]DAG were determined as described in Methods. Data shown as mean ± SEM values for triplicate determinations in a representative experiment. Similar observations were made in two other experiments. Total amount of labeled DAG measured in untreated cells were 4,460 ± 160 dpm (M-DAG), and 8,310 ± 370 dpm (A-DAG).

increases in both M-DAG ($p<0.05$) and A-DAG ($p<0.01$) were detected at 1000 U/ml, which increased to maximal levels at a GM-CSF concentration of 10,000 U/ml ($p<.025$ for M-DAG, $p<.005$ for A-DAG).

IL-3 also stimulated DAG generation in a dose-dependent fashion. At a concentration of 1,000 U/ml IL-3 singificantly elevated both M-DAG (Figure 4, $p<.05$) and A-DAG (Figure 4 inset, $p<.01$) which increased to optimal levels at 10,000 U/ml ($p<.005$ for both M-DAG and A-DAG).Thus both M-DAG and A-DAG generation exhibit the same sensitivity to concentration of either GM-CSF or IL-3.

DISCUSSION

We have reported in this study that both GM-CSF and IL-3 stimulate a transient rise in BMM DAG generation. Thus all three colony stimulating factors that stimulate DNA synthesis in BMM, namely CSF-1, GM-CSF and IL-3 elevate DAG generation in these cells (11). Considering that the agents TPA and exogenous PLC also promote DNA synthesis (2)

in the presence of DAG elevation (11), it may be postulated that this biochemical event may be an important mediator of macrophage proliferation. Non-mitogenic stimuli however have also been shown to elevate either, or both, M-DAG, or A-DAG levels in BMM (11). This would suggest that an elevation in DAG generation is perhaps a necessary event but not sufficient by itself for macrophage proliferation.

Although GM-CSF and IL-3 do promote DNA synthesis in BMM they are not as potent a stimulus as CSF-1 (2). In this study, however, we show that GM-CSF and IL-3 are as potent as CSF-1 in elevating the levels of both forms of DAG in BMM (11). CSF-1, GM-CSF and IL-3 also stimulate BMM Na^+, K^+ATPase activity to the same level (4). The dose-responses observed for DAG generation is within the same range as that reported for other events stimulated by GM-CSF and IL-3 in BMM, such as DNA synthesis (2), pinocytosis (8), plasminogen activator production (9), and Na^+, K^+ATPase activity (4).

Thus, although an increase in DAG generation may be associated with macrophage proliferation, it also correlates with other CSF-mediated events. Not only is this is the first report to suggest that DAG generation by GM-CSF may be associated with macrophage proliferation, but it is also the first to demonstrate an increase in DAG generation by IL-3, suggesting that this may be a signaling mechanism for this factor in BMM.

The phospholipid source(s) of CSF-stimulated M-DAG and A-DAG is currently under investigation, although the prior demonstration that inositol trisphosphate levels were not elevated by GM-CSF and IL-3 in BMM (10,6) suggests that DAG does not derive from phosphatidylinositol-4,5-bisphosphate hydrolysis. In human neutrophils it has been proposed that GM-CSF stimulates a phospholipase D-mediated hydrolysis of phosphatidyl choline yielding phosphatidic acid which is dephosphorylated to DAG (19). Whether or not this mechanism occurs in macrophages is presently being examined.

The effects of GM-CSF and IL-3 on BMM DNA synthesis, plasminogen activator activity and the stimulation of pinocytosis can be mimicked to some extent by the addition of TPA or exogenous PLC to these cells (2,6,9,8). This would suggest that the generation of DAG and the activation of PKC may be important in the mechanisms of action of these CSFs on these events. Both GM-CSF and IL-3 have been found to stimulate the phosphorylation of a PKC substrate protein in haemopoietic cell lines which proliferate in response to these CSFs (20,21). Atlhough IL-3 stimulates the translocation of PKC in FDC-P1 cells (22,23), it has never been shown that this event correlates with an elevation of DAG generation. This report is the first to demonstrate that DAG is a candidate second messenger for IL-3 action.

Thus, IL-3 and GM-CSF elevate DAG generation in BMM with similar kinetics to that observed for CSF-1 (11). We suggest that this event is important for macrophage proliferation, but is not sufficient on its own to initiate this cellular process. The mechanism

of DAG elevation in BMM may perhaps to be similar for all three CSFs, however this is to be further delineated by current studies in this laboratory.

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