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Granulocyte/macrophage colony-stimulating factor primes human neutrophils for increased diacylglycerol generation in response to chemoattractant

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Pretreatment of human neutrophils with granulocyte macrophage-colony stimulating factor (GM-CSF) augments several biological responses to chemoattractants (e.g. the respiratory burst, degranulation, and chemotaxis). However, little is known regarding the intracellular effects of priming with GM-CSF. In the present study, we have investigated the effects of GM-CSF on the generation of diacylglycerol (DAG), a proposed mediator of neutrophil responses. GM-CSF alone produced only a small increase in cellular DAG mass, which was most apparent after 30 min. GM-CSF pretreatment (60 min), however, caused a striking augmentation in DAG generation in response to the chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLP), compared with neutrophils preincubated without GM-CSF. The augmentation in DAG generation correlated with an enhancement by GM-CSF of superoxide generation in response to fMLP. The data suggest that GM-CSF may exert some of its biological effects by enhancing DAG generation in response to a second agonist.

Diacylglycerol; Granulocyte/macrophage colony-stimulating factor; Formyl-methionyl-leucyl-phenylalanine; Human neutrophil; Respiratory burst

1. INTRODUCTION

The neutrophil is the major phagocytic cell involved in the first line of defense against invading microorganisms. Recent studies have identified a family of proteins which stimulate the differentiation and proliferation of stem cells to form mature blood cells [1,2]. One of these, GM-CSF, stimulates the proliferation and differentiation of neutrophilic granulocytes and macrophages. GM-CSF also has a variety of effects on the function of mature cells. In neutrophils, these include an enhancement of the superoxide-generating respiratory burst induced by agonists such as fMLP [3,4]. However, the priming of the superoxide generation is slow, requiring 1-2 h of preincubation with GM-CSF. Additional metabolic effects of GM-CSF have been noted, including enhanced synthesis of platelet activating factor in response to chemoattractants [5]. GM-CSF may also function in vivo to enhance neutrophil responses; Baldwin et al. [6] reported that injected GM-CSF enhances the frequently depressed neutrophil functions in patients with AIDS.

Little is known about the signal transduction pathways involved in GM-CSF-primed neutrophil activation. GM-CSF alone does not stimulate the generation or release of superoxide, arachidonate, or inositol phosphates. G-proteins have recently been implicated in the early effects of GM-CSF in neutrophils which include pH changes, Na^+ influx, and gene expression [7,8]. Diacylglycerol has recently been implicated as a mediator of a variety of fMLP-activated neutrophil responses, including superoxide generation and degranulation [9,10]. In the present studies, we have tested the effect of GM-CSF on superoxide generation and diglyceride generation, the latter using a method which quantifies mass. GM-CSF produces only a small increase in basal DAG levels, but markedly enhances DAG generation in response to fMLP. Enhancement of DAG generation correlates with priming of fMLP-activated superoxide generation.

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2. MATERIALS AND METHODS

2.1. *Materials*

Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from American Hospital Supply Corp. Lymphocyte Separation Medium (6.2% Ficoll, 9.4% sodium diatrizoate) was purchased from Bionetics Laboratory Products. Octyl-β-D-glucopyranoside, adenosine-5'-triphosphate, fMLP, DL-dithiothreitol and fetal bovine serum were from Sigma. Recombinant human GM-CSF (200000 units/ml (5 μg/ml)) was purchased from AMGen biologicals. [γ-^{32}P]ATP (spec. act. 4000 Ci/mmol) was purchased from ICN Radiochemicals. Cardiolipin (bovine heart) was shipped on dry-ice from Avanti polar lipids. *E. coli* diglyceride kinase was obtained from Lipidex. Silica gel 60 F_{254} TLC plates were from EM Science. All other reagents and solvents were of the highest quality available commercially.

2.2. *Isolation of neutrophils*

Human neutrophils were isolated from the peripheral blood of

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healthy donors as described previously [11]. Isolated cells were suspended in PBS buffer which contained 5% fetal bovine serum and were used within 4 h [9,12].

2.3. *Neutrophil treatment and lipid extraction*

Cells (1×10^7 in 0.8 ml) were preincubated at 37°C for the indicated time either with or without GM-CSF in 13 × 100 mm polystyrene tubes, and were then stimulated with fMLP (1 μM). At appropriate times, incubations were terminated by transfer to 13 × 100 mm glass test tubes (with Teflon screw caps) containing 3 ml of chloroform/methanol (1:2, v/v). Lipids were extracted by the method of Bligh and Dyer [13]. Diacylglycerol was quantified by conversion to [^{32}P]phosphatidic acid using the method of Preiss et al. [14], with some modifications as described in [9]. Lipids were extracted, and [^{32}P]phosphatidic acid was separated by TLC and visualized by autoradiography. Phosphatidic acid-containing areas were scraped and counted as detailed previously [9,15].

3. RESULTS AND DISCUSSION

Weisbart and colleagues [4] previously showed that preincubation of neutrophils with GM-CSF augments superoxide generation in response to fMLP. We initially confirmed the priming effect of GM-CSF on superoxide generation. Neutrophils preincubated for 3 min and then stimulated with fMLP showed a respiratory burst rate of 4.6 nmol/min per 10^6 cells (table 1). After prolonged preincubation (30 and 60 min) at 37°C, fMLP-stimulated superoxide generation had declined slightly to about 2.5 nmol/min per 10^6 cells (table 1). In contrast, cells preincubated with 100 pM GM-CSF for either 30 or 60 min (but not 3 min) showed a 3–4-fold increase in respiratory burst rate compared with similarly preincubated controls (table 1). Longer preincubation did not result in a further enhancement of superoxide generation under these conditions (data not shown). Neutrophils primed with higher concentration of GM-CSF (200 pM) did not show any appreciable increase in respiratory burst rate (table 1). Neutrophils incubated with GM-CSF alone for up to 120 min did not produce any superoxide (data not shown). Thus, GM-CSF primes human neutrophils for an enhanced fMLP-stimulated respiratory burst.

Table 1

Effect of purified recombinant GM-CSF-priming on neutrophils respiratory burst

Incubation mixture	Time (min)	Stimulus	nmol superoxide/min per 10^6 cells
Cells + diluent	03	fMLP	4.6 ± 0.4
Cells + diluent	30	fMLP	2.5 ± 0.4
Cells + diluent	60	fMLP	2.6 ± 0.3
Cells + GM-CSF (100 pM)	03	fMLP	4.5 ± 0.3
Cells + GM-CSF (100 pM)	30	fMLP	8.4 ± 0.5
Cells + GM-CSF (100 pM)	60	fMLP	9.8 ± 0.6
Cells + GM-CSF (200 pM)	60	fMLP	10.4 ± 1.1

Superoxide generation was measured by monitoring cytochrome *c* reduction using an SLM/AmincoDW2000 spectrophotometer in the dual wavelength mode (A_{549} minus A_{540}) with constant slow stirring (Teflon-coated stir-bar) as described in [21]. Values are means ± SE of 3 observations using a single cell preparation. The experiment was repeated 3 times using 3 separate cell preparations

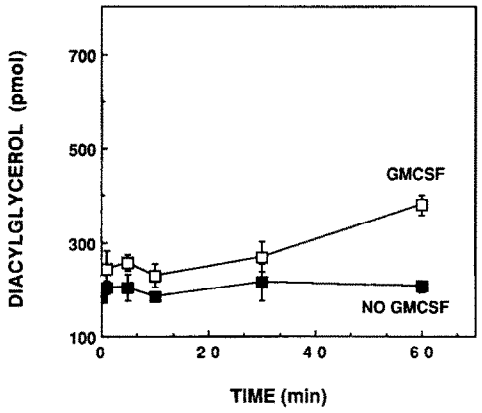

Fig.1. Time course for the production of diacylglycerol in GM-CSF (100 pM) treated (open squares) and untreated human neutrophils (filled squares). Cells were incubated at 37°C. Aliquots (10^7 cells/data point) were removed, and analyzed for DAG. For each time point, the mean ± SE of 3 incubations from same cell preparation are shown (DAG pmol/10^7 cells). Results are representative of two experiments using separate cell preparations.

We previously studied the generation of diacylglycerol in response to fMLP in neutrophils which had been primed with phorbol 12-myristate, 13-acetate, an activator of protein kinase C [9]. In those studies, priming with PMA resulted in increased DAG generation in response to fMLP, and the enhanced DAG generation correlated with the enhanced functional response. Herein, we have investigated the effects of GM-CSF on neutrophil DAG levels. We find that GM-CSF alone has only a small effect on basal levels of DAG, particularly at time points beyond 30 min (fig.1, open squares). In the absence of GM-CSF, basal DAG remained unchanged. Thus, GM-CSF produces a small increase above basal DAG levels, and this change may account for some of the early functional/biochemical changes seen with GM-CSF alone (e.g. GM-CSF is a weak secretagogue [16], and induces changes in sodium/proton antiport activity [8]).

The effect of GM-CSF pretreatment on DAG generation in response to a second stimulus was evaluated. Neutrophils primed with GM-CSF for 60 min and then stimulated with fMLP showed a rapid (60 s) and sustained (beyond 5 min) increase in diglyceride levels (fig.2, open squares). The amount of DAG generated was considerably larger than that produced in response to fMLP alone (filled squares) following preincubation for the same period without GM-CSF. Thus, priming with GM-CSF enhances the production of DAG in response to fMLP, and the enhanced DAG generation correlates with the augmentation of fMLP-activated superoxide generation.

The mechanism by which GM-CSF primes fMLP-activated DAG generation is uncertain, but is similar in some respects to priming by other agents. For example,

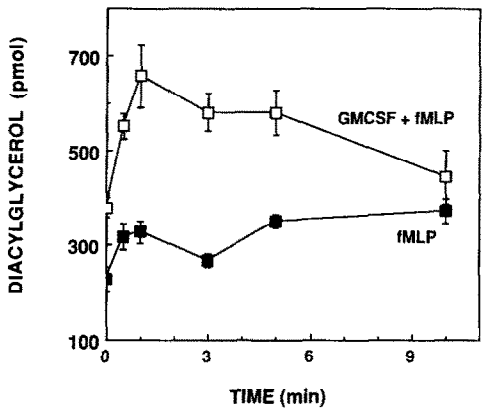

Fig.2. Time course for fMLP (1 μM)-stimulated DAG generation in cells preincubated for 60 min at 37°C with 100 pM GM-CSF (open squares) or parallel preincubated control cells (filled squares). Aliquots (10^7 cells/data point) were removed, and analyzed for DAG. For each time point, the mean ± SE of 3 incubations from same cell preparation are shown. Results are representative of two experiments using separate cell preparations.

a low dose of PMA which does not itself activate the respiratory burst, primes the burst towards a second stimulus such as fMLP [17]. We have shown that PMA priming is accompanied by augmented DAG generation in response to fMLP [9]. While low-dose PMA primes for responses to fMLP, it does not enhance superoxide generation when a second, high dose, of PMA is used. Similarly, GM-CSF fails to prime for an enhanced oxidative response to PMA [3]. Although the mechanism for neutrophil priming by GM-CSF is unknown, it is possible that the small enhancement of DAG generation at later times is acting via protein kinase C, similar to low doses of PMA [9]. The overall effect may relate to increased chemoattractant receptor number and/or receptor coupling to the phospholipase(s) which generate DAG. For example, low doses of PMA have been shown to mobilize an intracellular store of fMLP receptors, causing increased expression of fMLP receptors on the cell surface of human neutrophils [18,19]. Similarly, Weisbart et al. [20] showed that GM-CSF augments the number of GM-CSF receptors on the cell surface. Thus, although the time course is longer, priming by GM-CSF shows a number of similarities to priming by phorbol esters. In summary, the present studies show that GM-CSF causes a weak enhancement of basal DAG levels which occurs slowly (>30 min), and that it causes a significant enhancement of DAG generation in response to a chemoattractant.

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