Granulocyte/macrophage colony-stimulating factor affects *myo*-inositol metabolism in a novel manner

Implications for its priming action on human neutrophils

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Little is known about the signal transduction processes involved in the priming action of granulocyte/macrophage colony-stimulating factor (GM-CSF) on neutrophils. This study has used *myo*-[³H]inositol-labelled human neutrophils to determine whether preincubation with GM-CSF influences *myo*-inositol (Ins) metabolism in control cells, or in cells stimulated with the bacterial chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (fMetLeuPhe). GM-CSF pretreatment did not influence the total cellular ³H radioactivity content, demonstrating that the cytokine had no effect on Ins uptake. However, neutrophils pretreated with GM-CSF showed a dramatic 25–40% fall in the free [³H]Ins content of the cell, which was almost quantitatively recovered in a 2–4-fold increase in radioactivity within PtdIns. The remainder of the ³H radioactivity was found proportionately distributed throughout all other [³H]Ins-containing metabolites. Interestingly, in comparison with controls, the GM-CSF-stimulated increases in [³H]polyphosphoinositide (including 3-phosphorylated lipids) and [³H]Ins polyphosphate contents were consistently higher than that observed with PtdIns. This observation suggests that GM-CSF influences the hormone-sensitive pool of PtdIns, possibly through the activation of a PtdIns synthase which is rate-limiting to subsequent metabolic pathways. This is the first report of an action of GM-CSF on Ins metabolism, and highlights the conversion of Ins to PtdIns as a key regulatory metabolic step.

INTRODUCTION

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is one of a family of glycoproteins that are important in regulating the production, survival and maturation of haematopoietic precursors in the bone marrow. GM-CSF also regulates the activities of mature phagocytes by enhancing their phagocytic and bacteriocidal capabilities [1,2]. For example, exposure of neutrophils to GM-CSF has been shown to up-regulate the intercellular adhesion molecule CD11b [3], as well as increasing the surface expression of receptors of fMetLeuPhe [4]. Preincubation of neutrophils with GM-CSF also enhances the following responses to subsequent physiological stimuli: phagocytosis [5], arachadonic acid release [6], leukotriene synthesis [6,7], mobilization of intracellular Ca²⁺ [8], changes in internal pH [8], and the neutrophil superoxide-generating respiratory burst [9]. Such an action has been termed 'priming', since GM-CSF treatment alone has little, if any, effect. GM-CSF must therefore play an important role in neutrophil activation and host defence.

To date, the underlying signal transduction pathways involved in the priming action of GM-CSF on neutrophils are poorly understood, although one report concluded that *myo*-inositol (Ins) lipid metabolism was not involved [10]. However, a role of Ins metabolism in GM-CSF neutrophil priming was suggested by data which demonstrated that preincubation with GM-CSF increased the magnitude of Ca²⁺ transients evoked by several chemotactic factors, including fMetLeuPhe ([8]; C. H. Macphee & J. E. Merritt, unpublished work). Inositol lipid metabolism is known to be intimately involved in receptor-mediated Ca²⁺ mobilization. Furthermore, the observation that the priming ability of GM-CSF can be significantly reduced in pertussis toxin-treated neutrophils [10–12] implies an essential role for a G-protein in the cytokine's priming action. It is also noteworthy

that the responses of neutrophils to various Ca²⁺-mobilizing agonists are also acutely sensitive to pertussis toxin pretreatment [10,13–15]. Taken together, these data suggest that GM-CSF and the Ca²⁺-mobilizing agonists share the same G-protein to mediate their physiological functions. It was therefore hypothesized that GM-CSF mediates its effects, at least in part, through changes in inositol lipid metabolism.

This study has therefore used [³H]Ins-labelled human neutrophils to investigate whether GM-CSF pretreatment alters inositol lipid metabolism alone or in combination with fMetLeuPhe. The data demonstrate that GM-CSF affects Ins metabolism in a novel manner which could explain many, if not all, of its known priming actions.

MATERIALS AND METHODS

Materials

Percoll and dextran T500 were purchased from Pharmacia (Uppsala, Sweden); Hepes (ultrapure) and fMetLeuPhe were from Calbiochem; recombinant human GM-CSF was from R & D Systems, Inc (Minneapolis, MN, U.S.A.); *myo*-[2-³H]inositol, (15.4 Ci/mmol) was from NEN Research Products (Stevenage, Herts., U.K.). All organic solvents and other laboratory reagents were analytical grade.

Preparation and incubation of human neutrophils

Neutrophils were prepared from freshly drawn blood by dextran sedimentation of erythrocytes followed by Percoll density-gradient centrifugation to separate leucocytes as previously described [16]. Neutrophils [3–6 × 10⁸] were then washed twice by centrifugation (5 min, 300 g)/resuspension in a buffer containing 145 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 25 mM-Hepes (pH 7.4 at 37 °C), 10 mM-glucose and 1% (w/v) BSA. The cell pellet was then resuspended in 10 ml of buffer containing

Abbreviations used: GM-CSF, granulocyte/macrophage colony-stimulating factor; fMetLeuPhe, formyl-methionyl-leucyl-phenylalanine; PIC, phosphoinositidase C; CMP-PtdOH, cytidine monophosphoryl phosphatidate.

[^3H]Ins (0.5 mCi/ml; 15.4 Ci/mmol) and divided into 2×5 ml portions. The [^3H]Ins was routinely cleaned by pretreatment on a column of Dowex (formate) resin just prior to use. The two neutrophil suspensions were incubated for 150 min at 37 °C with intermittent shaking before 200 pM-GM-CSF or vehicle was added for a further 45 min. Cells were then washed by centrifugation/resuspension; twice with 1% BSA buffer and once with 0.1% BSA buffer. Finally, cell pellets were resuspended in 6.5 ml of 0.1% BSA buffer containing 1 mM- CaCl_2 . The presence of 200 pM-GM-CSF or vehicle was maintained throughout these procedures. Following a 5–10 min preincubation at 37 °C, 1 ml triplicate aliquots of the appropriate cell suspension were taken and incubated with either 0.1 μM -fMetLeuPhe or vehicle for 45 s. Incubations were terminated by the addition of an equal volume of ice-cold 7% (v/v) perchloric acid. The acid-soluble and -insoluble material was separated by centrifugation at 1500 *g* for 30 min at 4 °C.

Extraction and separation of inositol phosphates

The acid-soluble fraction, which contained the [^3H]inositol phosphates, were split into two portions and neutralized by extraction with tri-*n*-octylamine [17]. One set of triplicates was routinely analysed by anion-exchange chromatography on AG-1 (X8, 200–400 mesh, formate form) columns, as described by Downes *et al.* [17]. The other portions were pooled to maximize the recovery of incorporated radioactivity and resolved on a weak anion-exchange h.p.l.c. column (Partisphere WAX 5 μm particle size, in a 12.5 cm cartridge; Whatman). The h.p.l.c. column was eluted with a gradient of water (A) and 0.5 M- $(\text{NH}_4)_2\text{HPO}_4$ (pH 3.2, 25 °C; B) as follows: 0 min, 0% B; 7.5 min, 0% B; 15 min, 22% B; 90 min, 22% B; 95 min, 100% B; 103 min, 100% B; 105 min, 0% B. The flow rate was 1 ml/min and 308×0.35 ml fractions were collected. Radiolabelled InsP_3 , InsP_4 , InsP_5 and InsP_6 standards were kindly provided by Dr. L. Stephens, Department of Biochemistry, AFRC, Cambridge, U.K., and were used to characterize the elution profile.

Extraction and separation of inositol lipids

The acid-insoluble pellet was washed with ice-cold 3.5% HClO_4 containing 2 mM-EDTA before ^3H -labelled lipids were extracted and analysed by a procedure very similar to that described by Stephens *et al.* [18]. Briefly, washed pellets were solubilized by vigorous mixing with 2 ml of (0.1 M-HCl/0.1 mM-Ins/5 mM-EDTA/0.75 M-NaCl)/methanol/chloroform (4:10:5, by vol.). Two phases were obtained by the addition of 740 μl of chloroform and 740 μl of a solution containing 0.1 M-HCl, 0.1 mM-Ins and 5 mM-EDTA. After mixing and centrifugation, the upper phase was carefully aspirated and the lower and interphases were dried under vacuum prior to deacylation [18]. Glycerophospho-

inositol phosphates were separated using a Partisphere SAX anion-exchange h.p.l.c. column (Whatman) and eluted with a gradient of water (A) and 1.25 M- $(\text{NH}_4)_2\text{HPO}_4$ (pH 3.8, 25 °C; B) at a flow rate of 1 ml/min as follows: 0 min, 0% B; 5 min, 0% B; 45 min, 12% B; 60 min, 30% B; 61 min, 100% B; 65 min, 100% B; 66 min, 0% B. A radiolabelled glycerophosphoinositol 3-phosphate standard was prepared by deacylation of the product of a soluble 3-phosphatidylinositol kinase isolated from human placental tissue [18a]. In all cases the levels of ^3H -containing metabolites were determined by standard liquid scintillation counting.

Measurement of the neutrophil oxidative burst

In parallel experiments, the enhanced neutrophil respiratory burst in response to fMetLeuPhe was measured by luminol chemiluminescence [19]. Neutrophils (1×10^6 /ml) were preincubated with luminol (15 μg /ml) for 5 min at 37 °C in the presence of 1 mM- CaCl_2 prior to fMetLeuPhe addition. Chemiluminescence was monitored using a lumiaggregometer with continuous stirring.

RESULTS

Priming of the neutrophil oxidative burst by GM-CSF

Fig. 1 clearly demonstrates that preincubation with GM-CSF (200 pM) enhanced fMetLeuPhe (0.01–1 μM)-stimulated superoxide production when compared with controls. This effect was evident even at a concentration of fMetLeuPhe (0.01 μM) which had very little effect when added to control cells. To investigate

Fig. 1. Effect of GM-CSF pretreatment on superoxide anion production by neutrophils in response to fMetLeuPhe

Freshly prepared human neutrophils were preincubated with GM-CSF (200 pM) or vehicle for 45 min at 37 °C prior to fMetLeuPhe (0.01–1 μM) addition. The arrows indicate the time of addition of fMetLeuPhe, and the respiratory burst was measured by luminol chemiluminescence as described in the Materials and methods section. The data are from a representative experiment which has been repeated on four separate occasions with essentially the same findings.

Table 1. Effect of GM-CSF preincubation on fMetLeuPhe-stimulated [^3H]Ins phosphate metabolism: Dowex separation

Freshly prepared human neutrophils, loaded with [^3H]Ins and pretreated with GM-CSF (200 pM) or vehicle, were stimulated with 100 nM fMetLeuPhe for 45 s. [^3H]Ins phosphates were extracted and separated on Dowex anion-exchange columns as described in the Materials and methods section. Data are expressed as means $\pm$ S.E.M. for triplicate incubations from a representative experiment which has been repeated four times.

Pretreatment	Stimulus	$10^{-3} \times ^3\text{H}$ (d.p.m.)				
		InsP_1	InsP_2	InsP_3	InsP_4	$\text{InsP}_{5/6}$
Control	Control	45.0 $\pm$ 0.2	4.4 $\pm$ 0.4	2.1 $\pm$ 0.07	0.46 $\pm$ 0.07	0.12 $\pm$ 0.03
Control	fMetLeuPhe	63.3 $\pm$ 1.3	11.3 $\pm$ 0.3	8.1 $\pm$ 0.16	4.2 $\pm$ 0.38	0.25 $\pm$ 0.04
GM-CSF	Control	77.5 $\pm$ 3.8	10.5 $\pm$ 0.7	5.0 $\pm$ 0.38	1.8 $\pm$ 0.06	0.24 $\pm$ 0.05
GM-CSF	fMetLeuPhe	143.8 $\pm$ 4.3	39.2 $\pm$ 3.0	24.3 $\pm$ 0.29	14.5 $\pm$ 0.77	0.47 $\pm$ 0.09

Table 2. Effect of GM-CSF preincubation on fMetLeuPhe-stimulated [³H]Ins phosphate metabolism: h.p.l.c. separation

Samples were prepared as described in Table 1 and h.p.l.c. analysis was performed as outlined in the Materials and methods section. Material from triplicate incubations was pooled prior to h.p.l.c. to maximize recovery of radioactivity. The data compare results from two different experiments and are presented as total d.p.m. obtained for each metabolite. Numbers in parentheses indicate the fold increase in radioactivity in response to fMetLeuPhe for each pretreatment.

Expt.	Pretreatment	Stimulus	10 ⁻³ × ³ H (d.p.m.)				
			Free Ins	InsP ₁ /InsP ₂	InsP ₃	InsP ₄	InsP _{5/6}
A	Control	Control	43 171	44.5	3.0	1.9	0.9
	Control	fMetLeuPhe	43 131 (1)	138.4 (3.1)	27.0 (9.0)	19.9 (10.5)	18.9 (20.3)
	GM-CSF	Control	32 265	121.8	12.1	7.7	8.7
	GM-CSF	fMetLeuPhe	32 462 (1)	310.6 (2.6)	81.8 (6.8)	65.1 (8.5)	62.3 (7.2)
B	Control	Control	53 228	48.5	2.1	0.5	1.6
	Control	fMetLeuPhe	53 568 (1)	111.6 (2.3)	12.4 (5.9)	11.7 (23.9)	1.7 (1.1)
	GM-CSF	Control	31 325	146.9	13.0	7.1	6.6
	GM-CSF	fMetLeuPhe	31 439 (1)	475.3 (3.2)	81.2 (6.3)	65.9 (9.3)	7.1 (1.1)

Table 3. Effect of GM-CSF preincubation on fMetLeuPhe-stimulated [³H]inositol lipid metabolism

Incubation conditions were identical to those described in Table 1, but in this case phospholipids were extracted, deacylated and subjected to h.p.l.c. analysis as outlined in the Materials and methods section. Data are expressed as the means ± s.e.m. for triplicate incubations from a representative experiment (Expt. A of Table 2) which has been performed on four separate occasions with similar findings. *P < 0.05 for fMetLeuPhe versus control incubations.

Pretreatment	Stimulus	10 ⁻³ × ³ H (d.p.m.)					
		PtdIns	PtdIns3P	PtdIns4P	PtdIns(3,4)P ₂	PtdIns(4,5)P ₂	PtdIns(3,4,5)P ₃
Control	Control	2831 ± 35	3.00 ± 0.06	119.2 ± 2.6	0.29 ± 0.02	94.4 ± 4.7	0.25 ± 0.04
Control	fMetLeuPhe	2672 ± 147	2.90 ± 0.03	122.1 ± 3.8	1.20 ± 0.07*	77.7 ± 4.0*	0.53 ± 0.04*
GM-CSF	Control	6143 ± 283	9.80 ± 0.33	381.9 ± 18.6	1.30 ± 0.05	263.1 ± 10.6	1.00 ± 0.07
GM-CSF	fMetLeuPhe	6002 ± 96	9.60 ± 0.08	355.4 ± 11.1	3.90 ± 0.22*	230.3 ± 12.9*	2.30 ± 0.38*

whether this priming effect of GM-CSF could be mediated through changes in Ins metabolism, cells were loaded with [³H]Ins and the levels of metabolites were analysed.

Inositol phosphate metabolism

The data in Tables 1 and 2 confirm and extend previous findings that fMetLeuPhe stimulates significant changes in inositol phosphate metabolism [20,21]. Table 1 shows data obtained from Dowex elutions, whereas Table 2 gives data from h.p.l.c. separations. It should be noted that the data in Table 1 and Expt. A of Table 2 were derived from the same samples. H.p.l.c. analysis allowed free Ins to be measured (i.e. ³H radioactivity not retarded by the column) in addition to a more accurate recovery and resolution of inositol phosphates, in particular InsP_{5/6} (Table 2). Results showed that incubation with fMetLeuPhe (100 nM) for 45 s increased the cellular ³H content of all of the inositol phosphates measured (InsP₁–InsP_{5/6}; Tables 1 and 2). In addition, GM-CSF-pretreated neutrophils demonstrated a significantly enhanced rate of [³H]Ins incorporation into every inositol phosphate when compared with controls (Tables 1 and 2). GM-CSF preincubation, however, appeared to have little effect on the fMetLeuPhe-induced changes in [³H]InsP₁, [³H]InsP₂ and [³H]InsP₃ metabolism when compared with control pretreated cells, as the percentage increase in the incorporated radioactivity for each metabolite was very similar between the two treatments (Table 2). As this simple relationship was not sustained for fMetLeuPhe-stimulated changes in InsP₄ and InsP_{5/6} metabolism (Table 2), a possible influence of GM-CSF on the metabolism of these inositol polyphosphates cannot be excluded. Finally, in stark contrast to the observations on enhanced rates of radio-

activity incorporation, GM-CSF treatment caused a dramatic 25–40% fall in the free [³H]Ins content of neutrophils (Table 2).

An additional observation that was independent of GM-CSF addition was a variable fMetLeuPhe-stimulated increase in cellular [³H]InsP_{5/6} content (Table 2; Expt. A versus Expt. B). Although fMetLeuPhe-stimulated changes in InsP₁ to InsP₄ were relatively consistent between different neutrophil preparations, the same could not be said for fMetLeuPhe-stimulated InsP_{5/6} metabolism. It was found that with half of the neutrophil preparations fMetLeuPhe induced a marked increase in [³H]InsP_{5/6} levels (e.g. Expt. A), whereas the remainder (e.g. Expt. B) showed very little, if any, enhanced response (Table 2).

Inositol lipid metabolism

Table 3 shows that fMetLeuPhe addition causes significant changes in neutrophil inositol lipid metabolism, including the newly described PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. The data presented in this paper demonstrate that, following incubation with fMetLeuPhe for 45 s, the [³H]PtdIns(4,5)P₂ content fell by approx. 15%, whereas the cellular content of both [³H]PtdIns(3,4)P₂ and [³H]PtdIns(3,4,5)P₃ increased by around 3- and 2-fold respectively (Table 3). The identity of the 3-phosphorylated lipids was assumed from the recent literature which has described the structural identification of two novel inositol lipids whose levels are transiently increased in fMetLeuPhe-stimulated neutrophils [22,23]. The rate of incorporation of [³H]Ins into all other inositol-containing lipids was unaffected by fMetLeuPhe addition. Again, the extent of these fMetLeuPhe-stimulated changes in inositol lipid metabolism was not significantly altered by preincubation with GM-CSF. How-

Table 4. GM-CSF influences human neutrophil [^3H]Ins metabolism independently of [^3H]Ins uptake

The data represent the total d.p.m. in each ^3H -containing metabolite for the two experiments described in Table 2. The total cellular ^3H radioactivity content for each incubation was obtained by adding the d.p.m. in all of the inositol-containing metabolites.

Expt.	Pretreatment	Stimulus	$10^{-3} \times ^3\text{H}$ (d.p.m.)				Total
			Free Ins	PtdIns	Inositol phosphates	Other phosphoinositides	
A	Control	Control	43 171	8 493	50	651	52 365
	Control	fMetLeuPhe	43 131	8 016	204	613	51 964
	GM-CSF	Control	32 265	18 429	150	1 971	52 815
	GM-CSF	fMetLeuPhe	32 462	18 006	520	1 805	52 793
B	Control	Control	53 228	6 687	53	300	60 268
	Control	fMetLeuPhe	53 568	6 717	138	320	60 743
	GM-CSF	Control	31 325	25 062	174	1 770	58 331
	GM-CSF	fMetLeuPhe	31 439	24 165	630	1 609	57 843

Table 5. GM-CSF stimulates the incorporation of [^3H]Ins into PtdIns at a lower rate than that observed for other inositol-containing metabolites

The data shows ratios obtained by dividing the incorporated ^3H radioactivity from GM-CSF-pretreated cells by that of controls for all of the phosphoinositides plus the InsP_3 pool.

Expt.	GM-CSF-treated/control						
	PtdIns	PtdIns3P	PtdIns4P	PtdIns(3,4) P_2	PtdIns(4,5) P_2	PtdIns(3,4) P_3	InsP_3
A	2.2	3.3	3.2	4.5	2.8	4.0	4.0
B	3.7	5.8	6.4	5.6	4.4	6.0	6.3

ever, in keeping with the findings on inositol phosphate metabolism, the ^3H content of all inositol lipids was significantly increased in GM-CSF-pretreated neutrophils (Table 3).

GM-CSF affects neutrophil [^3H]Ins metabolism

The data presented thus far can be summarized and clarified by totalling the radioactivity found in all of the inositol-containing metabolites for each individual experiment (Table 4). Importantly, preincubation with GM-CSF did not influence the total [^3H]Ins content of the cell, demonstrating that the cytokine had no effect on Ins uptake. The changes observed upon GM-CSF preincubation could not therefore be explained by a change in specific radioactivity of the intracellular [^3H]Ins due to an artefact of the labelling procedure. This, of course, assumes that there was no change in the synthesis of endogenous Ins from glucose 6-phosphate over the incubation period. Table 4 also clearly demonstrates that when a short-term [^3H]Ins labelling protocol is used, free Ins and PtdIns always comprise greater than 95% of the cell-associated ^3H radioactivity. Indeed, the dramatic 25–40% fall in the free [^3H]Ins content observed with GM-CSF-treated neutrophils was almost quantitatively recovered in a 2–4-fold increase in radioactivity in [^3H]PtdIns (Table 4).

In an attempt to further characterize the effect of GM-CSF on Ins metabolism, the enhanced ^3H radioactivity contents of various inositol-containing metabolites from cells pretreated with GM-CSF were divided by the appropriate radioactivity found in control cells. Table 5 shows the calculated ratios obtained for all of the inositol lipids plus the InsP_3 pool. It was noteworthy that the GM-CSF-stimulated increases in [^3H]polyphosphoinositide (including 3-phosphorylated lipids) and [^3H]Ins P_3 contents were consistently higher than that observed for the increase in [^3H]PtdIns. The implication is that only a specific pool of PtdIns was affected by GM-CSF addition.

DISCUSSION

Extensive evidence from a number of laboratories has demonstrated that GM-CSF acts as a priming agent on mature neutrophils, enhancing superoxide anion generation and other responses to a second stimulus such as fMetLeuPhe [5–9]. However, little is known about the mechanism of action of this cytokine. This current study has clearly shown that GM-CSF has a major effect on Ins lipid metabolism. The effect appears to be specifically focused on the conversion of free Ins to PtdIns. This is an intriguing finding, as the cytokine has apparently isolated a metabolic step which has not been previously considered as a key regulatory point in what is universally accepted as an important cycle in the hormonal control of cell function [24,25].

In evaluating the data from the present study, one must first consider the implications of the labelling protocol utilized. As a short-term (3 h) [^3H]Ins labelling regime was used, alterations in radioactivity do not reflect changes in mass of the metabolite, but in fact measure changes in specific radioactivity. Thus three different explanations could account for the manner by which GM-CSF augments the conversion of free [^3H]Ins to [^3H]PtdIns: (1) enhanced PtdIns/Ins base exchange; (2) increased PtdIns synthase activity; and (3) enhanced PtdIns phospholipid transfer protein activity. Although an Ins headgroup exchange mechanism exists in cells [26], this route can be excluded from further consideration by data from a study which used neutrophils labelled with [^{32}P]P $_i$ [12]. It was found that GM-CSF increased the rate of ^{32}P incorporation into the acid-insoluble fraction of neutrophils independently of enhanced cellular P $_i$ uptake. The majority of label incorporated over the short incubation period used in that study would be predicted to be located in the phosphoinositides. As the [^{32}P]PtdIns formed could only be derived from cytidine monophosphoryl phosphatidate (CMP-PtdOH), then an action of GM-CSF on the Ins headgroup

Fig. 2. Proposed site of action for GM-CSF enhancement of the responses to Ca^{2+} -mobilizing agonists such as fMetLeuPhe

The interaction of GM-CSF with its receptor on neutrophils leads to the activation of a specific rate-limiting PtdIns synthase and/or PtdIns transfer protein. The resulting increased synthesis of PtdIns is confined to the receptor-sensitive pool of inositol lipids which allows a greater and more efficient supply of $\text{PtdIns}(4,5)\text{P}_2$, the precursor molecule to the two second messengers, $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol (DAG). G, pertussis toxin-sensitive G-protein; fMLP, fMetLeuPhe

exchange reaction can be ruled out. The most obvious effector candidates for GM-CSF's actions are a PtdIns synthase and/or a PtdIns transfer protein. Evidence does exist for a specific plasma membrane form of PtdIns synthase which could function in the recycling of a hormone-sensitive pool of inositol lipids [27]. Similarly, the well-documented occurrence of a PtdIns transfer protein could facilitate the rapid equilibration of this lipid species between subcellular compartments [28]. The knowledge that GM-CSF increases the phosphotyrosine content of several proteins in neutrophils [12] lends support to the possibility that at least one of the aforementioned processes could be regulated in such a manner.

It was interesting that GM-CSF increased the specific radioactivity of PtdIns to a consistently lower level (approx. 3-fold) than that observed for other inositol-containing metabolites (approx. 5-fold; Table 5). This has two important ramifications. First, only a specific pool of PtdIns was affected by GM-CSF, and secondly, all other labelled metabolites must, in the main, be derived from this precursor PtdIns pool. This is in keeping with current evidence which suggests that one pool of PtdIns may provide the substrates for hormone-stimulated inositol phospholipid breakdown, and is characterized by a high metabolic turnover rate, whilst the bulk of cellular PtdIns turns over more slowly and is not immediately affected by hormonal stimulation [29]. Thus a short-term labelling protocol, like the one employed in the present study, would be expected to preferentially label the hormone-sensitive inositol lipid pool. One conclusion, therefore, which could be readily derived from the data presented here, is that GM-CSF exerts all of its action on the receptor-sensitive pool of PtdIns at a step which is rate-limiting to all subsequent metabolic pathways. One possible consequence would be that the mass of this PtdIns pool would increase. If this step was indeed rate-limiting, the receptor-sensitive inositol lipid cycle, in response to GM-CSF, would become more highly tuned, allowing a more efficient supply of $\text{PtdIns}(4,5)\text{P}_2$. This is the substrate which is cleaved by a hormone-sensitive inositol lipid-specific phospholipase C (phosphoinositidase C; PIC) to produce the ubiquitous second messengers $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. Fig. 2 shows a schematic diagram of how GM-CSF could act to influence the hormone-sensitive inositol lipid pathway.

From this action, one would predict that when GM-CSF-

treated cells are stimulated with a given concentration of Ca^{2+} mobilizing agonist, a greater outflow of second messengers should result. Interestingly, this is exactly what has been documented. Naccache and colleagues [8] clearly demonstrated that GM-CSF augmented the $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} transients produced in response to three different Ca^{2+} -mobilizing agonists. Moreover, GM-CSF pretreatment has also been observed to cause a striking enhancement in diacylglycerol mass in response to fMetLeuPhe [30]. This GM-CSF-dependent augmentation in diacylglycerol levels may well explain many priming actions of this cytokine via an enhanced activation of at least one of the isoenzymes of protein kinase C present within neutrophils. This is in keeping with the observation that priming by GM-CSF shows marked similarities to priming by activators of protein kinase C. For example, cell-permeant activators of protein kinase C have been shown to prime neutrophils for increased superoxide generation [31] plus arachidonic acid release and leukotriene synthesis [32].

This current model (Fig. 2) of how GM-CSF acts to prime neutrophils is also totally consistent with the observation that its priming capability is inhibited by pertussis toxin [10–12]. A pertussis toxin-sensitive G-protein has been demonstrated to be intimately involved in the coupling of Ca^{2+} -mobilizing receptors to PIC in neutrophils [13–15]. Thus, according to the postulate derived from the present study, one would accurately predict that GM-CSF's priming action would be pertussis toxin-sensitive. The proposed mechanism is also consistent with the observation that preincubation with GM-CSF augmented the respiratory burst to NaF and guanosine 5'-[α -thio]triphosphate, two agents that directly activate G-proteins [33]. That particular study ruled out the possibility that priming by GM-CSF involved regulation at the level of the cell surface receptor.

One study has stated that the priming mechanism of GM-CSF is not associated with phosphatidylinositol turnover [10]. This conclusion arose from data demonstrating that the '% increase' in [^3H]Ins incorporated into InsP_3 in response to fMetLeuPhe was no different between control and GM-CSF-pretreated cells. No crude d.p.m. levels were presented. However, as demonstrated in Tables 1 and 2, the fMetLeuPhe-stimulated '% increase' in [^3H]Ins incorporated into InsP_3 does not alter between the different incubation conditions, but the net incorporation of [^3H]Ins was considerably greater in GM-CSF-pretreated neutrophils.

Two other observations were noted in this current study. The first was that GM-CSF enhanced the rate of incorporation of label into the newly described 3-phosphorylated inositol lipids to a similarly elevated level as it did with the other polyphosphoinositides (Table 5). This could result from the fact that the 3-phosphorylated lipids are derived from the same receptor-sensitive pool of PtdIns. This metabolic relationship has been strongly suggested by recent data by Stephens *et al.* [23] which outlined the synthetic pathway of $\text{PtdIns}(3,4,5)\text{P}_3$ in human neutrophils. The second observation was the curious, albeit variable, GM-CSF-independent effect of fMetLeuPhe in rapidly increasing the [^3H]Ins $\text{P}_{5/6}$ content of neutrophils (Table 2). fMetLeuPhe has previously been shown to rapidly elevate $\text{InsP}_{5/6}$ levels in the promyelocytic HL60 cell line, but what function this serves remains obscure [34]. The fact that this response appeared to be all or nothing (Table 2) may suggest that these highly phosphorylated inositol phosphates have a specific role which is dependent on the particular functional state of the neutrophil at a given time.

In conclusion, the data presented in this study have provided evidence that GM-CSF may prime human neutrophils by increasing the synthesis of a specific pool of PtdIns which is rate-limiting to subsequent receptor-sensitive pathways. Further work

is required to determine if this effect involves a specific PtdIns synthase or PtdIns transfer protein.

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