Involvement of a Phospholipase D in the Mechanism of Action of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF): Priming of Human Neutrophils In Vitro With GM-CSF Is Associated with Accumulation of Phosphatidic Acid and Diradylglycerol

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Summary

The generation of diradylglycerol (DRG) and phosphatidic acid (PdtOH) was investigated in neutrophils primed with granulocyte-macrophage colony-stimulating factor (GM-CSF). Mass accumulation of DRG and PdtOH was measured using reversed-phase high performance liquid chromatography and thin layer chromatography, respectively. GM-CSF had no direct effect on the levels of PdtOH and DRG, but it increased PdtOH generation and the late phase of DRG accumulation in human neutrophils stimulated with FMLP. The elevation of the mass of PdtOH peaked ~100 s and clearly preceded that of DRG, which peaked at 150 s. The diacylglycerol kinase inhibitor R59022 enhanced the sustained increase in DRG but did not produce a parallel inhibition in PdtOH production. GM-CSF was without effect on the level of inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] and did not affect the liberation of Ins(1,4,5)P_3 induced by FMLP. These findings exclude the involvement of the PtdIns(4,5)P_2-specific phospholipase C/diacylglycerol pathway in the sustained phase of DRG accumulation. The early (30-s) appearance of PdtOH clearly suggests that GM-CSF enhanced FMLP receptor–linked phospholipase D (PLD) generation of PdtOH. PLD was assessed more directly by formation of labeled phosphatidylethanol (PEt) through PLD capacity of catalyzing a *trans*-phosphatidylation in presence of ethanol. The formation of PEt associated with a concomitant decrease in PdtOH directly demonstrated that the mechanism by which GM-CSF enhances PdtOH production is activation of a PLD active on phosphatidylcholine. This study provides evidence that the mechanism of action of GM-CSF involves upregulation of PLD activity leading to enhanced generation of PdtOH and DRG in FMLP-stimulated neutrophils. These findings may provide the basis for several of the priming effects of GM-CSF.

Granulocyte-macrophage CSF (GM-CSF)[1], a glycoprotein of 22 kD, is released by several cell types, including T lymphocytes, fibroblasts, eosinophils, monocytes/macrophages, and neutrophils. Though GM-CSF has first been found to promote the growth and the differentiation of myeloid progenitors to both granulocytes and monocytes, it has also been shown to influence the responsiveness of mature human phagocytes, in particular, neutrophils, to different stimuli (1–3). Surface expression of functional antigens such as GFA-1 and GFA-2, as well as the adhesion molecules Mo-1 and Leu-M5 is increased by GM-CSF in the absence of a subsequent stimulation (4, 5). Preincubation of neutrophils with GM-CSF is associated with a rapid increase in the number of low affinity FMLP receptors expressed on the cell surface (6), with a cytosolic alkalinization and a stimulation of membrane-bound GTPase activity (7). GM-CSF-pretreated neutrophils show enhanced responses to soluble stimuli, such as FMLP, leukotriene B_4 (LTB_4), and platelet-activating factor (PAF). The neutrophil responses shown to be augmented in GM-CSF-treated cells include superoxide production (6), lysosomal enzyme release (4), calcium mobilization (8), and arachidonic acid release and metabolism through the 5-lipoxygenase

[1] *Abbreviations used in this paper:* DMF, dimethylformamide; DRG, diradylglycerol; GM-CSF, granulocyte-macrophage CSF; HSA, human serum albumin; LTB_4, leukotriene B_4; PdtOH, phosphatidic acid; PEt, phosphatidylethanol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; RT, room temperature.

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pathway (9, 10). GM-CSF also augments neutrophil phagocytosis (11) and the concomitant synthesis of LTB_4 (12).

Although high affinity binding sites for GM-CSF have been demonstrated (13), little is known about the signal transduction pathway(s) involved in the actions of GM-CSF. A growing body of evidence implicates guanine nucleotide regulatory proteins in coupling receptor occupancy to the activation of the phospholipase C (PLC) pathway in neutrophils (14, 15). PLC activation produces two putative second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which releases Ca^{2+} from intracellular stores, and diglycerides, which act as endogenous activators of protein kinase C (PKC) (16). Recent data demonstrated the involvement of guanine binding proteins in neutrophil activation and priming by GM-CSF (7, 17), as well as tyrosine phosphorylation of proteins, including the *ras* gene product P21 (2), a GTP binding protein. However, more recent reports dissociated the mechanism of action of GM-CSF from direct stimulation of the phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] hydrolysis pathway and PKC activation (18, 19). On the other hand, it has been suggested that GM-CSF may act through the modulation of the generation of cyclic nucleotides (19).

Another potential signaling pathway in cell activation involving a phospholipase D (PLD) has recently been studied in various cell types, including human phagocytes and the HL-60 human promyelocytic cell line (20–22). The activation of PLD may involve a guanine nucleotide binding protein (23). It gives rise to choline-linked phosphoglycerides breakdown products, namely choline and phosphatidic acid (PdtOH), with diacyl-PdtOH and 1-0-alkyl-2-acyl-PdtOH species (21, 22). PLD activation also leads to the formation of the corresponding diradylglycerides (DRG) through a phosphatidate phosphohydrolase activity (24).

To gain insight into the mechanisms whereby GM-CSF enhances neutrophil functional responsiveness, we have studied its effects on the phospholipase pathway(s) involved in neutrophil activation. Our findings indicate that pretreatment of the cells with GM-CSF leads to enhanced accumulation of PdtOH and DRG upon simulation with FMLP, and strongly suggest that this effect is mediated by an upregulation of PLD activity.

Materials and Methods

Reagents. FMLP, 4-dimethylaminopyridine, triethylamine, PLD (Cabbage type I), phosphatidylcholine (dioleoyl), phosphatidic acid dipalmitoyl, 1,3-distearoyl-glycerol (1,3-distearin), 1,2-distearoyl-rac-glycerol, 1,2-dipalmitoyl-sn-glycerol, 1.2-dioleoyl-sn-glycerol, 1-stearoyl-2-arachidonoyl-sn-glycerol, and coomassie brilliant blue (CBB R-250) were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-dilinoleoyl-rac-glycerol was from Nu Check Prep Inc., (Elysian, MN). 1-0-hexadecyl-2-oleoyl- and 1-0-hexadecyl-2-palmitoyl-sn-glycerol were prepared by PLC (type XIII; Sigma Chemical Co.) digestion of the corresponding phosphatidylcholines (Sigma Chemical Co.). 1-0-[^{3}H]alkyl-2-acetyl-sn-glycero-3-phosphocholine (132–179 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). α-naphthyl-isocyanate and 1,8-diazacyclo-[5,4,0]-undec-7-ene were from Aldrich Chemical Co. (Milwaukee, WI). Dimethylformamide was from Pierce Chemical Co. (Rockford, IL). Silica gel 60 thin layer chromatography (TLC) plates were from Merck & Co. (Darmstadt, FRG). R59022 was bought from Janssen Life Sciences (NJ). HBSS was from Gibco Laboratories (Burlington, Ontario), and all solvents were HPLC grade from Anachemia (Montréal, Québec). Biosynthetic human recombinant GM-CSF (The Genetics Institute, Cambridge, MA) was resuspended in sterile buffered saline containing 0.01% human serum albumin (HSA); its stock solution (at 100 nM) was stored at −20°C and handled aseptically.

Neutrophil Preparation. Neutrophil suspensions were prepared as previously described (25). Briefly, venous blood collected on citrate dextrose phosphate adenine anticoagulant solution from healthy volunteers was centrifuged (250 *g*, 10 min), and the platelet-rich plasma was discarded. After dextran sedimentation, PMN were obtained by centrifugation over a Ficoll-Paque cushion. Contaminating erythrocytes were removed by hypotonic lysis, and purified neutrophils (98% neutrophils) were finally resuspended in HBSS containing 10 mM Hepes and 1.6 mM Ca^{2+}, pH 7.4. Cells were counted (model ZM; Coulter Electronics Inc., Hialeah, FL) and diluted in HBSS to a final concentration of 2×10^7/ml. Cell viability, as determined by trypan blue dye exclusion, was always >96% at the end of each experiment.

Incubation Conditions. Neutrophils (2×10^7/ml) were preincubated at room temperature (RT; 20°C) for the desired period of time with or without 400 pM GM-CSF (a concentration of GM-CSF previously described to induce optimal priming effects on neutrophil functions; see references 8 and 12). The cell suspensions were then warmed to 37°C for 5 min before FMLP challenge. At selected times after the addition of FMLP, the reactions were stopped by vortexing 4-ml aliquots of neutrophil suspensions with 7.2 ml of ice-cold chloroform/methanol/HCl (10 N) (100:200:2; vol/vol/vol). Control experiments used unstimulated neutrophils preincubated with or without GM-CSF. In the experiments with the DRG kinase inhibitor R59022, neutrophils were preincubated 10 min at 37°C with 20 μM R59022 before the addition of FMLP, as described (26).

Sample Preparation for DRG and PdtOH Analysis. Lipids were extracted by the Bligh and Dyer's technique (27), with modification. Briefly, after vortexing the cell suspension with cold chloroform/methanol/HCl (10 N) (as described above), samples were mixed with chloroform (0.6 ml/ml of the original cell solution) containing 1 μg of 1,3-distearin as an internal standard. After centrifugation (750 *g*, 10 min at 4°C), the chloroform layer was carefully removed, and the aqueous layer was vortexed with 2 ml chloroform. The combined chloroform fractions were washed with 1 ml of 0.1 N HCl and dried under nitrogen. The lipid samples were then dissolved in chloroform/methanol (1:2; vol/vol).

TLC of Lipids. Separation of the different neutral lipids was achieved using silica gel-60 plates previously developed in chloroform/methanol (1:2; vol/vol) for cleaning purposes. The elution solvent was made up of benzene/chloroform/methanol (80:15:3.25; vol/vol/vol) (system I). After evaporation of organic solvents under nitrogen, the plates were slightly stained with coomassie brilliant blue. The plates were then dried, the areas containing the diglyceride subclasses were scraped off, and the silica gel was collected. The DRG were eluted from the silica gel with 8 ml hexane/ether (25:75; vol/vol). The samples were dried under nitrogen, and the DRG were either converted to their naphthylurethane derivatives for subsequent separation and quantitation by HPLC (see below) or to their anthracene-propionyl derivatives for further analysis by mass spectrometry (see below).

PdtOH were resolved from other lipids by TLC on silica gel-60 using a solvent containing chloroform/pyridine/88% formic acid

(50:30:7; vol/vol/vol) (system II). In some experiments, after an initial development of the TLC plate in system I, the phospholipids that remain at the origin were scraped off and recovered from the silica gel by sequential elution with 5 ml chloroform/MeOH (1:2; vol/vol) and 2 ml MeOH, and separated using solvent system II to resolve PdtOH.

Analysis of DRG: HPLC Separation and Quantitation of DRG. DRG samples purified by TLC with solvent system I and recovered from TLC plates were prepared for analysis using a slight modification of Rustow's method to obtain naphthylurethane derivatives (28). Briefly, dried DRG samples were dissolved in 100 μl dimethylformamide (DMF). 5 μl of α-naphthylisocyanate (a large excess of the reagent) and 10 μl of 1,8-diazacyclo-[5,4,0]-undec-7-ene (0.1 M solution in DMF) were added simultaneously to each sample. The stoppered glass vials were heated at 85°C under nitrogen for 30 min. After cooling to RT, excess reagent was destroyed by addition of 500 μl of MeOH for 10 min, and the reaction mixture was then centrifuged (250 *g*, 5 min at RT). The clear supernatant was carefully removed, and the precipitated material was then resuspended in 0.6 ml ether and centrifuged at 250 *g*. The clear supernatants were combined and mixed with 10 ml of solvent A (acetonitrile/water, 75:25; vol/vol) and applied to Sep Pak C_{18} cartridges (Waters Associates, Milford, MA) prewashed with 15 ml solvent A. The samples were then washed with 5 ml solvent A, followed by 10 ml acetonitrile/water (90:10), and the derivatized DRG were eluted with 5 ml of distilled *n*-hexane. The derivatized DRG samples were dried and dissolved in isopropanol for HPLC analysis. Sep Pak cartridges were then washed with 4 ml isopropanol and reequilibrated with solvent A for further use. Separation and quantitation of neutrophil DRG was achieved using a Radial Pak C_8 cartridge (5 × 100 mm, 10-μm particles; Waters Associates) protected by a C_8 guard cartridge (Pierce Chemical Co.). Derivatized DRG were eluted at a flow rate of 1.5 ml/min using two solvent mixtures: solvent A-1, acetonitrile/water (70:30; vol/vol) and solvent A-2 (100% acetonitrile), and the following program: step 1, 0–5 min, isocratic in A-1; step 2, 5–5.5 min, 100% A-1 to 33% A-1/67% A-2; step 3, 5.5–11.5 min isocratic in 33% A-1/67% A-2; step 4, 11.5–13 min from 33% A-1/67% A-2 to 5% A-1/95% A-2; step 5, 13–16 min, isocratic in 5% A-1/95% A-2; step 6, 16–16.1 min from 5% A-1/95% A-2 to 100% A-2; step 7, 16.1–26 min, isocratic in 100% A-2; step 8, 26–26.1 min from 100% A-2 to 100% A-1; step 9, 26.1–36.5 min isocratic in 100% A-1. DRG species were detected by absorbance at 223 nm using a variable wavelength UV photometer. The products were identified on the basis of their coelution with authentic standards. Quantitation of DRG was performed by comparison of the peak areas with that of the internal standard 1,3-distearin; quantitative values given for DRG are the sum of all DRG species appearing on the HPLC tracings. Those conditions of analysis using UV detection at 223 nm allowed a detection limit of 5–10 pmol DRG.

Analysis of PdtOH. Samples prepared for PdtOH analysis (using TLC with solvent system II, see above) were stained with coomassie brilliant blue, as described by Nakamura and Handa (29). Briefly, TLC plates were immersed in a 0.03% solution of the dye in 30% MeOH containing 100 mM NaCl, for 30 min, and destained for 5 min in 30% MeOH, 100 mM NaCl. The plates were air dried and scanned using an image analysis system (Amersham Corp.). Integration of thin layer bands was performed using GL-1000 software supplied with the image analyzer (Amersham Corp.). Quantitation of PdtOH was achieved using a standard curve (0.75, 1.5, and 3 μg) dipalmitoyl phosphatidate obtained for each analysis. In some experiments, the fatty acid composition of TLC-purified PdtOH was studied by GC-MS using the procedure described above for DRG species. Alkyl-PdtOH were assessed as alkyl-lyso-PdtOH after alkaline hydrolysis of TLC-purified PdtOH species. Briefly, PdtOH were hydrolyzed with 80% methanolic NaOH (1 N) overnight at RT, and the base hydrolysis products were extracted using the Bligh and Dyer's technique (27). The organic phase was then dried and analyzed on silica gel using solvent system II. Lyso-alkyl-PdtOH were quantitated using charring densitometry (30) and lyso-PdtOH for standard curve.

Preparation of Phosphatidylethanol (PEt). PEt was prepared from phosphatidylcholine (dioleoyl) by *trans*-phosphatidylation with cabbage PLD. Cabbage PLD (50 U) dissolved in 1 ml of 10% ethanol, 40 mM calcium chloride, pH 5.6, and 400 μg of phosphatidylcholine dissolved in 1 ml of water-saturated diethylether were incubated overnight at RT. The lipids were extracted with diethylether/ethanol (4:1; vol/vol), and the organic phase was dried under nitrogen before being separated by TLC on silica gel-60 plates in chloroform/methanol/acetic acid (65:15:2; vol/vol/vol) (21). PEt was localized by coomassie brilliant blue staining (29) and identified by comparison to published Rf values (21).

PEt Biosynthesis in Neutrophils. Neutrophils (10^7) were prelabeled with 1-0-[^{3}H]alkyl-2-acetyl-sn-glycero-3-phosphocholine. 1-0-[^{3}H]alkyl-2-acetyl-sn-glycero-3-phosphocholine (10 μCi/ml, in HBSS containing 2.5 mg HSA was added to the cell suspension at the final concentration of 15 nM (2 μCi/ml), and the cells were incubated at 37°C for 90 min. Cells were washed twice in HBSS buffer containing 0.5 mg/ml HSA and resuspended in the same buffer (without HSA) to a final concentration of 10^7/ml. Cells were treated with 400 pM GM-CSF or diluent, as described above. The cell suspensions were then warmed to 37°C for 5 min before FMLP challenge in the absence or presence of 0.5% ethanol added 1 min before the agonist. Lipids were extracted as described above and the lipids were resolved by TLC on silica gel-60 plates as before. Standard lipids were located by coomassie brilliant blue staining and plates were scraped in 5-mm slices and assayed for radioactivity.

Inositol 1,4,5-Trisphosphate (Ins(1,4,5)P_3) Measurement by RIA. Ins(1,4,5)P_3 was assayed in incubation media with a commercial RIA kit (Amersham Corp.). Briefly, neutrophils (10^7/ml) were preincubated for 1 h at 37°C with or without 400 pM GM-CSF before stimulation with 1 μM FMLP. Incubations were terminated by addition of 1 vol ice-cold TCA (15%). After sedimentation of precipitated material, supernatants were extracted with 10 vol of water-saturated diethyl ether and then titrated to pH 7.5 with $NaHCO_3$. The amounts of Ins(1,4,5)P_3 in neutralized superna-

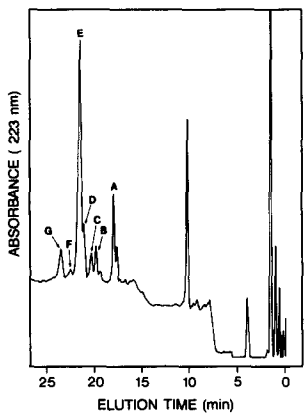

Figure 1. Reverse-phase HPLC elution profile of naphthylurethane derivatives of standard diglycerides. Standard DRG were converted to their naphthylurethane derivatives, purified and separated by RP-HPLC, as described in Materials and Methods. 20–300 pmol of each DRG standard was applied on the column: *A*, 1,2-diacyl $C_{18:2}/C_{18:2}$; *B*, 1,2-diacyl $C_{18:0}/C_{20:4}$; *C*, 1,2-diacyl $C_{18:1}/C_{18:1}$; *D*, 1-0-alkyl-2-acyl $C_{16:0}/C_{16:0}$; *E*, 1-0-alkyl-2-acyl $C_{16:0}/C_{18:1}$; *F*, 1,2-diacyl $C_{18:0}/C_{18:0}$ (arises from isomerization of the 1,3-diacyl $C_{18:0}/C_{18:0}$ standard [peak G]).

tants were assessed using Amersham Corp.'s specified assay procedures.

Statistical Analysis. Results are expressed as the mean ± SEM of at least five experiments. Statistical analysis was performed by student's paired *t* test, and significance was considered attained when p was <0.05, one-tailed.

Results

Influence of GM-CSF on DRG Levels in Human Neutrophils. Separation and quantitation of human neutrophils DRG were performed using a sensitive HPLC-UV method that did not require radiolabeling. Standards of various DRG species were separated as naphthylurethane derivatives by HPLC (Fig. 1). The elution times of DRG naphthylurethane derivative standards were dependent on the degree of fatty acid unsaturation and carbon chain length. The 1,2-diacyl ($C_{18:2}/C_{18:2}$ derivative eluted first (Fig. 1, peak *A*) and was followed by 1,2-diacyl $C_{18:0}/C_{20:4}$ (peak *B*), 1,2-diacyl $C_{18:1}/C_{18:1}$ (peak *C*), 1-0-alkyl-2-acyl $C_{16:0}/C_{16:0}$ (peak *D*), 1-0-alkyl-2-acyl $C_{16:0}/C_{18:1}$ (peak *E*), 1,2-diacyl $C_{18:0}/C_{18:0}$ (peak *F*), and 1,3-diacyl $C_{18:0}/C_{18:0}$ (peak *G*). Using this HPLC system, naphthylurethane derivatives of DRG obtained from neutrophils eluted between 17 and 25 min of the 36.5-min elution program (Fig. 2). The limit of detection for DRG naphthylurethanes, as assessed with 1,2-dioleoylglycerol at 223 nm, was 5–10 pmol. The identification of peaks in biological sample chromatograms was made by comparison of retention times with those of DRG standards. We used 1,3-distearin as an internal standard to monitor DRG recovery throughout the extraction procedures and conversion into naphthylurethane derivatives. The 1,3-distearin naphthylurethane standard eluted as two peaks (Fig. 1, peaks *F* and *G*; Fig. 2, peaks *8* and *9*). In fact, TLC analysis of the commercial 1,3-distearin standard revealed the presence of the 1,2-distearin isomer, which eluted as peak *F* of Fig. 1 in HPLC analysis. In contrast, we did not observe the presence of 1,3-DRG isomers in TLC analysis of biological DRG samples. Recovery of the internal standard at the level of HPLC analysis was found to be 30–50%.

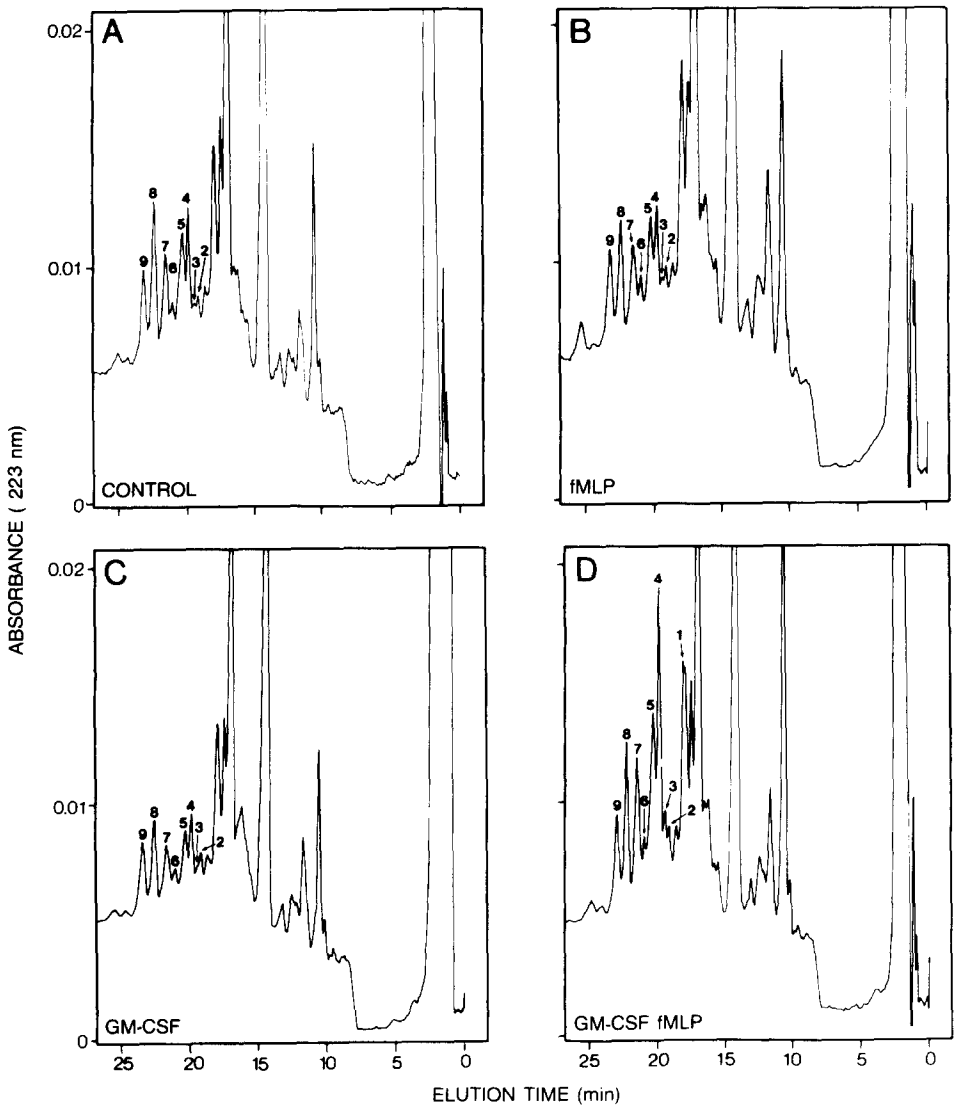

Figure 2. Reverse-phase HPLC elution profiles of naphthylurethane derivatives of endogenous DRG from normal human blood neutrophils. Neutrophils (2×10^7/ml) were preincubated for 1 h at RT without GM-CSF (*A* and *B*) or in presence of 400 pM GM-CSF (*C* and *D*) before stimulation with 1 μM FMLP for 2.5 min at 37°C (*B* and *D*). The DRG from 5×10^7 cells were separated by TLC on Silica gel using solvent system (1), converted to their naphthylurethane derivatives and separated by RP-HPLC, as described in Materials and Methods. Peaks were identified by comparison of their elution times with those of derivatized standards (see Fig. 1). Peak *1* corresponded to 1,2-diacyl $C_{18:2}/C_{18:2}$ standard; peaks *2* and *3* were not identified; peaks *4–9* corresponded to 1,2-diacyl $C_{18:0}/C_{20:4}$, 1,2-diacyl $C_{18:1}/C_{18:1}$, 1-0-alkyl-2-acyl $C_{16:0}/C_{16:0}$, 1-0-alkyl-2-acyl $C_{16:0}/C_{18:1}$, 1,2-diacyl $C_{18:0}/C_{18:0}$, and 1,3-diacyl $C_{18:0}/C_{18:0}$, respectively. Chromatograms *A*, *B*, *C*, and *D* are derived from one representative experiment (cells were from the same donor); this experiment was repeated five times with similar results.

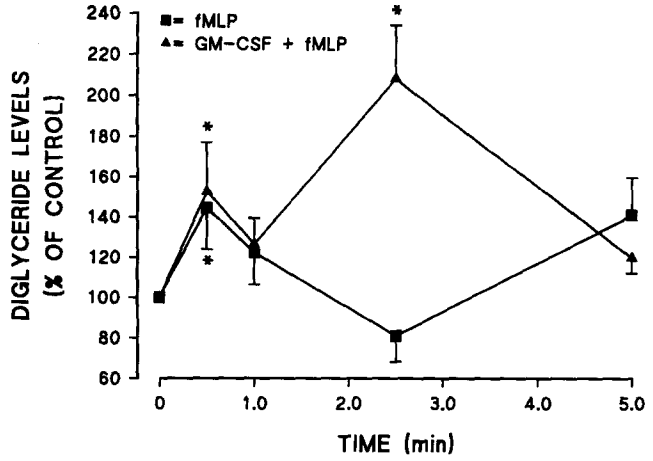

Figure 3. Effect of GM-CSF on the time course of DRG production in human neutrophils stimulated by FMLP. Neutrophils were preincubated at RT for 1 h with or without 400 pM or GM-CSF before stimulation with 1 μM FMLP for 2.5 min at 37°C. The endogenous DRG were purified and derivatized into naphthylurethane derivatives before RP-HPLC, as described in Materials and Methods. The amount of the DRG species formed at each time point was measured and the total amount was calculated. The results show the mean ± SEM of five experiments. The asterisks indicate that $p < 0.05$. The control values obtained at 0 min in unstimulated neutrophils were used to calculate the percentage and the p values shown.

HPLC analysis of DRG species also showed some qualitative limitations; for example, compounds $C_{18:0}/C_{20:4}$ (Fig. 1, peak *B*) and $C_{16:0}/C_{16:0}$ were found to have identical retention times in the system used.

sn-DRG Level in Neutrophils. Human neutrophils, incubated with or without 400 pM GM-CSF for 1 h at RT, showed similar levels of DRG (sum of all species detected), e.g., 167 ± 36 and 159 ± 29 pmol/10^7 neutrophils, respectively (n = 5), as assessed by HPLC analysis and correction for recovery using the internal standard. Similarly, we did not detect changes in the basal levels of DRG upon incubation of the cells with GM-CSF, over a 30-min period at 37°C (data not shown). These data indicated that GM-CSF did not induce by itself the formation of DRG.

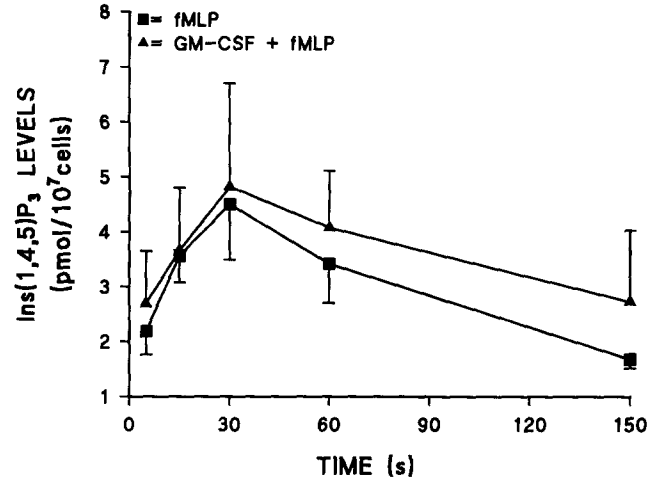

Figure 4. Effect of GM-CSF on the time course of $Ins(1,4,5)P_3$ production in FMLP-stimulated human neutrophils. Neutrophils (10^7/ml) were preincubated with or without 400 pM GM-CSF for 1 h at RT before stimulation with 1 μM FMLP at 37°C. $Ins(1,4,5)P_3$ levels were measured by RIA after extraction, as described in Materials and Methods. The results are expressed as the mean ± SEM of five experiments.

Fig. 3 shows the kinetics of the changes in DRG levels in FMLP-stimulated neutrophils. When neutrophils were stimulated with 1 μM FMLP, the levels of DRG rapidly increased by 45% within 30 s ($p < 0.05$), then dropped back to basal levels by 2.5 min. This was followed by a slower rise of DRG at 5 min (final level, 141% of basal). Neutrophils treated with GM-CSF exhibited two phases of DRG accumulation when challenged with FMLP (Fig. 3). The most striking effect of GM-CSF pretreatment was a second phase of DRG accumulation, which was maximal at 2.5 min before dropping back to basal levels by 5 min (Figs. 2 *D* and 3). The mean change (percent of control) of that second peak was 208 ± 26% ($p < 0.05$), ranging from 135 to 293%. The first peak of DRG accumulation in GM-CSF-treated neutrophils stimulated with FMLP reached 153% ($p < 0.05$) of the basal DRG levels. While there was no difference in the level of the first peak (30 s) of DRG accumulation in control or GM-CSF-treated cells, the levels of DRG at 2.5 min were significantly elevated in cells pre-exposed to the growth factor as compared with control cells.

Effect of GM-CSF on Ins(1,4,5)P_3 Formation in Human Neutrophils. The binding of FMLP to its receptor in neutrophils leads to phospholipase C-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (31). We examined the effect of GM-CSF on the formation of Ins(1,4,5)P_3, a breakdown product of PtdIns(4,5)P_2 hydrolysis. Basal levels of Ins(1,4,5)P_3 in unstimulated neutrophils were unchanged by GM-CSF treatment. Neutrophil stimulation with FMLP was associated with a rapid (30 s) and transient production of Ins(1,4,5)P_3 (Fig. 4); Ins(1,4,5)P_3 levels increased from a mean baseline of 2.2 ± 0.3 pmol/10^7 cells to a mean maximal level of 4.5 ± 1.0 pmol/10^7 cells. Neither the time course nor the maximal production of Ins(1,4,5)P_3 in FMLP-stimulated neutrophils were altered by GM-CSF treatment. Under those conditions, GM-CSF treatment increased the FMLP-induced O_2^- production by 170 ± 12% (n = 3). It is noteworthy that the kinetics of accumulation of Ins(1,4,5)P_3 and DRG in neutrophils stimulated with FMLP (Figs. 3 and 5) were very similar.

Influence of GM-CSF on the Levels of PdtOH in Human Neutrophils. The stimulation of neutrophils by FMLP led to a slight but nonsignificant increase of the level of PdtOH within 1 min 45 s (Fig. 5). GM-CSF, by itself, did not affect the basal levels of PdtOH in unstimulated neutrophils (data not shown). However, the addition of FMLP to GM-CSF-treated neutrophils resulted in a rapid and significant increase of the levels of PdtOH, which was detectable as early as 30 s and remained stable for up to 5 min (Fig. 5). Under these conditions, PdtOH increased from 0.24 ± 0.07 μg to 0.99 ± 0.15

Table 1. *Changes in Diglyceride Subspecies as a Function of Time in GM-CSF-treated Neutrophils Stimulated with FMLP*

	Time				
Peak	0	0.5	1	2.5	5
			min		
1	ND	ND	ND	ND	ND
2	1	1.36 ± 0.3	1.23 ± 0.3	1.74 ± 0.3	1.31 ± 0.4
3	1	2.19 ± 0.7	1.34 ± 0.3	3.40 ± 0.7*	1.05 ± 0.1
4	1	1.67 ± 0.3*	1.30 ± 0.2	3.07 ± 0.9*	1.34 ± 0.1*
5	1	1.35 ± 0.2	1.04 ± 0.1	1.82 ± 0.2*	1.15 ± 0.1
6	1	1.48 ± 0.2*	1.00 ± 0.1	1.89 ± 0.2*	0.98 ± 0.1
7	1	1.33 ± 0.2*	1.00 ± 0.2	1.92 ± 0.2*	1.16 ± 0.1

To calculate changes in diglyceride subspecies, an arbitrary unit of 1 was given to each subspecies (control at 0 min). Data represent the mean ± SEM of five experiments.
* $p < 0.05$.

$\mu g/10^7$ neutrophils at 1 min 45 s. At each time studied after FMLP challenge, the levels of PdtOH in GM-CSF-treated neutrophils were consistently two- to threefold higher than those of cells that had prior stimulation with FMLP (t = 0 min; Fig. 5).

Preincubation of GM-CSF-treated neutrophils with R59022, a DRG kinase inhibitor, was associated with a significant enhancement of both phases of FMLP-induced accumulation of DRG (Fig. 6). In contrast, preincubation of neutrophils with 20 μM R59022 did not significantly alter

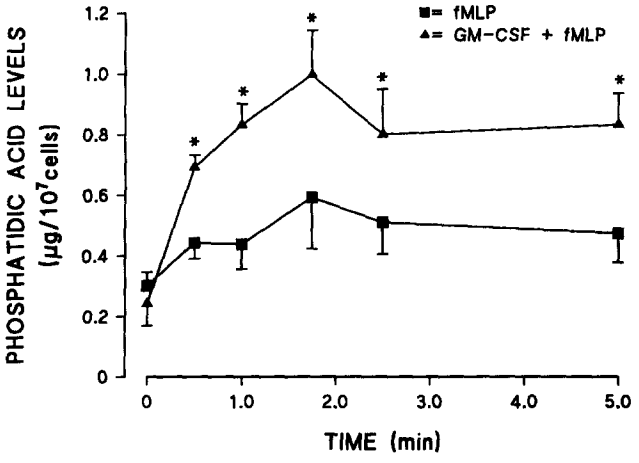

Figure 5. Time course of the formation of PdtOH in FMLP-stimulated neutrophils. Neutrophils (2×10^7/ml) were preincubated with or without 400 pM GM-CSF for 1 h at RT before stimulation with 1 μM FMLP at 37°C. PdtOH from 3.5×10^7 cells were separated from other lipids by TLC and quantified by densitometry after coomassie brilliant blue staining, as described in Materials and Methods. Data represent the mean ± SEM of four experiments. The asterisks indicate that $p < 0.05$.

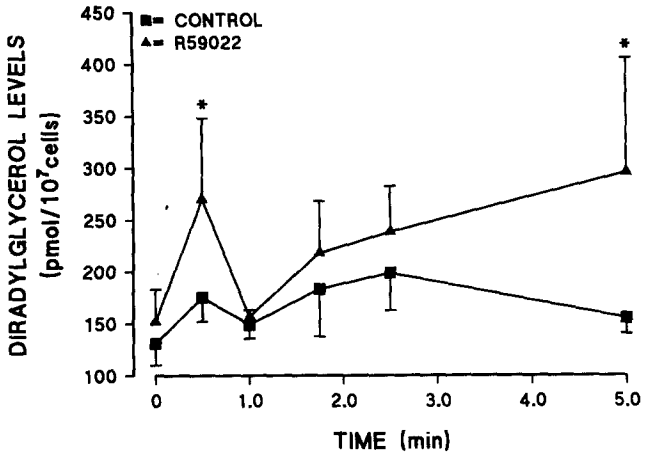

Figure 6. Effect of R59022 on the time course of the production of DRG in GM-CSF-treated neutrophils. Neutrophils were preincubated with 400 pM GM-CSF for 1 h at RT and incubated 10 min with or without 20 μM R59022 at 37°C before stimulation with 1 μM FMLP at 37°C. Endogenous DRG from 8×10^7 cells were purified by TLC, derivatized before RP-HPLC analysis, as described in Materials and Methods. DRG were calculated as described in the Fig. 3 legend. Results show the mean ± SEM of five experiments. The asterisks indicate that $p < 0.05$.

the time course of accumulation of PdtOH in GM-CSF-treated neutrophils stimulated with FMLP (Fig. 7). Basal levels of DRG and PdtOH in unstimulated neutrophils pretreated with GM-CSF were unchanged by preincubation of cells with R59022. Taken together, these observations are consistent with a major contribution of a PLD in the generation of PdtOH induced by GM-CSF treatment.

The alkyl-acyl composition of PdtOH was studied after basic hydrolysis under mild conditions (80% methanolic 1 N NaOH; 15 h at RT). The resulting products were extracted and purified by TLC. The levels of alkyl-PdtOH were not detectable in control cells nor in unstimulated cells treated with GM-CSF using charring densitometry (results not shown). However, when GM-CSF-treated neutrophils were stimulated with FMLP, alkyl-PdtOH rapidly increased to a detectable level and reached a plateau (0.06 $\mu g/10^7$ neutrophils) corresponding to 5–10% of the total PdtOH content within 60 s (Fig. 8). However, since recovery of a standard was not included, the values may underestimate the amount of alkyl-PdtOH.

Effect of GM-CSF on PEt Formation in Human Neutrophils. Advantage was taken of property of PLD-catalyzed formation of PEt by transphosphatidylation reaction in the presence of ethanol to assess the effect of GM-CSF on PLD activity. When 1-0-[^{3}H]alkyl-2-acetyl-sn-glycero-3-phosphocholine was added for 90 min to human neutrophils, 85 ± 2% was taken up by the cells. 88 ± 2% (n = 3) of the total cell radioactivity was in alkyl-acyl-phosphatidylcholine. Stimulation of these prelabeled neutrophils with FMLP led to a production of [^{3}H]alkyl-PdtOH. The level of [^{3}H]alkyl-PdtOH peaked within 1 min (two- to threefold) and declined slowly thereafter (data not shown). The most striking effect of GM-CSF

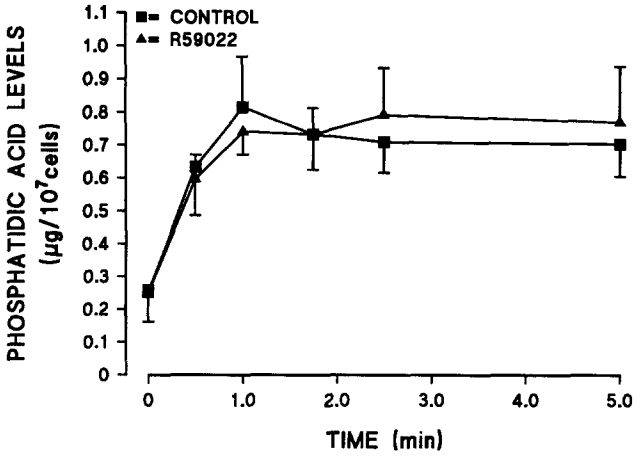

Figure 7. Effect of R59022 on the time course of the production of PdtOH in GM-CSF-treated neutrophils. Neutrophils were preincubated with or without 400 pM GM-CSF for 1 h at RT and incubated 10 min with 20 μM R59022 at 37°C before stimulation with 1 μM FMLP at 37°C. PdtOH from 3.5 × 10^7 neutrophils were purified by TLC and quantified by densitometry after coomassie brilliant blue staining, as described in Materials and Methods. Data represent the mean ± SEM of four experiments.

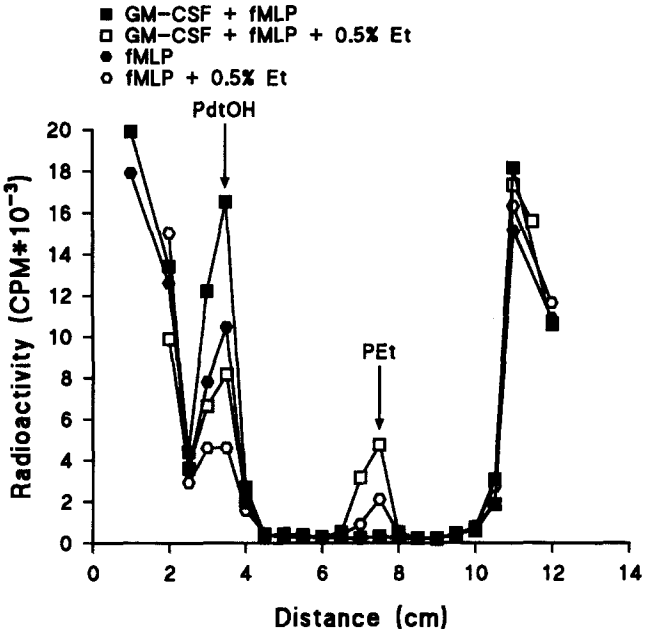

Figure 9. Formation of PdtOH and PEt in human neutrophils. Human neutrophils were labeled with 1-0-[^{3}H]alkyl-2-acetyl-sn-glycero-3-phosphocholine, as described in Materials and Methods. Cells (10^7/ml) were pretreated with or without GM-CSF (400 pM) for 1 h at RT before stimulation with 1 μM FMLP at 37°C in the presence or absence of 0.5% ethanol. Lipids were extracted and separated by TLC, and standard lipids were located by coomassie brilliant blue staining. Plates were scraped in 5-mm slices and assayed for radioactivity. Shown is a single experiment representative of three different ones.

pretreatment was an accumulation of [^{3}H]alkyl-PdtOH in FMLP-stimulated neutrophils (Fig. 9). Addition of 0.5% ethanol to control or GM-CSF-treated neutrophils 1 min before FMLP challenge led to a 50–60% inhibition of [^{3}H]alkyl-PdtOH accumulation. Inhibition of [^{3}H]alkyl-PdtOH formation was correlated with the formation of a novel [^{3}H]-labeled product (Rf = 0.5) with Rf similar to the standard PEt. Formation of this product, identified as [^{3}H]alkyl-PEt (21), was increased 2.4-fold in neutrophils pretreated with GM-CSF (Fig. 9). These data demonstrate that GM-CSF up-regulates a phosphatidylcholine-specific PLD under these conditions.

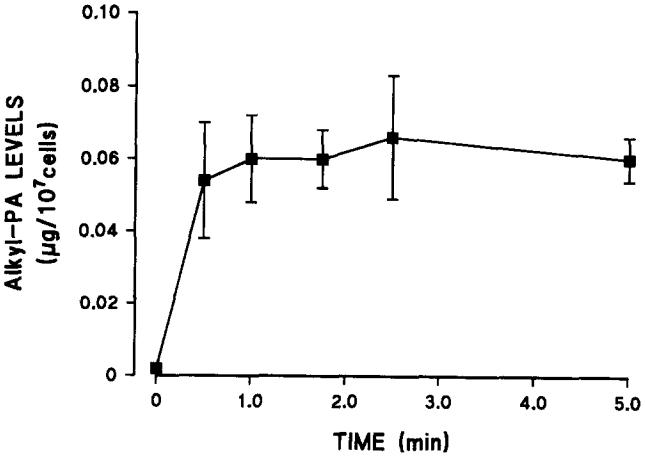

Figure 8. Time course production of alkyl-PdtOH in GM-CSF-pretreated neutrophils. PdtOH from 1.8 × 10^8 neutrophils treated for 1 h at RT with 400 pM GM-CSF and stimulated with 1 μM FMLP at 37°C was submitted to base hydrolysis. Base hydrolysis products were extracted and separated by TLC. Alkyl-lyso-PdtOH were quantitated by charring densitometry using a standard curve obtained with lyso-PdtOH. Data are the mean ± SD of two separate experiments.

Analysis of DRG Species in Neutrophils. Besides providing a mean for quantitation of DRG, HPLC also enabled comparison of the various DRG species formed under the different experimental conditions tested. HPLC analysis of the DRG in control neutrophils showed four principal DRG peaks (Fig. 2 *A*; peaks *4*, *5*, *6*, and 7). Peak 4 coeluted with $C_{18:0}/C_{20:4}$ and $C_{16:0}/C_{16:0}$ AAG standards (Fig. 1, peak *B*). Peaks 5, 6, and 7 comigrated with diacyl $C_{18:1}/C_{18:1}$, 1-0-alkyl-2-acyl $C_{16:0}/C_{16:0}$, and 1-0-alkyl-2-acyl $C_{16:0}/C_{18:1}$ DRG standards, respectively (Fig. 1, peaks *C*, *D*, and *E*). The DRG species eluting as peaks 2 and 3 have not yet been identified. Peak 1 had a retention time identical to that of $C_{18:2}/C_{18:2}$ (Fig. 1, peak *A*). The profile of DRG species in neutrophils was unchanged by preincubation with GM-CSF (Fig. 2 *C*). Similarly, we did not detect significant changes in the profile of DRG after a 2.5-min stimulation with FMLP (Fig. 2 *B*). In contrast, cells treated with GM-CSF exhibited major changes in several DRG species after 2.5-min stimulation with FMLP, with a significant increase of peaks 1, 3, and 4 (Fig. 2 *D*). Peak 4 showed the highest enhancement, averaging 307 ± 89% (n = 5) of its basal level. Peak 1, which eluted just after a contaminant, was detected only in GM-CSF-pretreated neutrophils stimulated with FMLP. As function of time, changes in DRG levels were due to a major contribution of peaks 1, 3, and 4.

Discussion

In the present study, we investigated the effects of GM-CSF on DRG and PdtOH levels in resting and stimulated neutrophils. It is important to point out that the present investigation relied on mass measurements, in contrast to previous studies on DRG and PdtOH in human neutrophils, which were carried out with cells prelabeled with radioisotopes (22, 31). It is indeed uncertain that true isotopic equilibrium of membrane lipids was reached by the short periods of radiolabeling used in those studies (32). Therefore, it seems that direct mass measurement is likely to provide a more reliable determination of the changes in lipid species.

We demonstrated herein that in human neutrophils pretreated with GM-CSF, the chemotactic peptide FMLP induced a rapid accumulation of PdtOH, and secondarily of DRG. GM-CSF treatment had no direct effect on the levels of DRG, PdtOH, and Ins(1,4,5)P_3 in unstimulated neutrophils, and affected neither the FMLP-stimulated formation of Ins(1,4,5)P_3 nor the initial peak of DRG accumulation.

Activation of neutrophils by chemoattractants is dependent upon GTP-binding proteins coupled to a PLC specific for PtdIns(4,5,)P_2 (33). Hydrolysis of PtdIns(4,5)P_2 leads to a transient rise in Ins(1,4,5)P_3 and a biphasic increase in diacylglycerol (34–36). The second phase of DRG increase observed in cytochalasin B–pretreated neutrophils stimulated with FMLP is correlated with optimal activation of the respiratory burst (35), and unlike the initial increase, was apparently derived from a phospholipid other than PtdIns (4,5)P_2 (36). Our findings clearly ruled out a PtdIns(4,5)P_2-specific PLC/diacylglycerol pathway in the mechanism of GM-CSF action, since the concomitant early peaks in Ins(1,4,5)P_3 and DRG were not altered by the cytokine in FMLP-stimulated neutrophils. Using [^{3}H]inositol-labeled neutrophils, Coffey et al. (19) reported similar results. Several lines of evidence also dissociate the late increase in DRG from the PtdIns (4,5)P_2-specific PLC pathway in cells treated with GM-CSF, and it has been suggested that DRG can be generated by PLC hydrolysis of a phospholipid other than PtdIns(4,5)P_2 or by another pathway (22, 31). Indeed, both PMA and dioctanoylglycerol induced the accumulation of DRG (1-0-alkyl-2-acylglycerol [EAG] and 1-2-diacylglycerol [AAG]) in human neutrophils without phosphoinositide hydrolysis and calcium mobilization (36–39). In several cell types, the PMA-stimulated release of DRG and choline metabolites was consistent with the PLC-mediated hydrolysis of phosphatidylcholine (40, 41). Recent evidence has implicated a PLD in the formation of PdtOH in chemoattractant-stimulated HL-60 cells and neutrophils (20–22, 31). Stimulation of 1-0-alkyl-[^{32}P]-PC-labeled cells with FMLP or PMA was found to induce the generation of alkyl-PdtOH from alkyl-phosphatidylcholine, most likely through a PLD activity (21). In addition, the recent demonstration of DRG formation with concomitant phosphorylcholine turnover in human PMN is consistent with an activation of a PLD activity (42).

In GM-CSF-treated cells stimulated with FMLP, a threefold increase in PdtOH preceded the twofold increase in DRG (Figs. 2, 3, and 5). Under these conditions, the mass of PdtOH was consistently four- to fivefold higher than the mass of DRG. Although PdtOH accumulation preceded the sustained phase of DRG formation in GM-CSF-treated neutrophils challenged with FMLP, it remains possible that PdtOH could be derived from a rapid phosphorylation of the DRG pool. If so, PdtOH accumulation should have been inhibited by the DRG kinase inhibitor R59022 (43). We found that R59022 increased the late phase of DRG accumulation, but was without significant effect on the time course of formation and accumulation of PdtOH. These observations indicated that most of the PdtOH pool is generated through the activation of a PLD. In addition, our findings of an increased proportion of base-stable PdtOH, identified as alkyl-PdtOH, in GM-CSF-treated neutrophils challenged with FMLP suggest that, since neutrophils do not contain alkyl-PtdIns(4,5)P_2, a phosphatidylcholine-specific PLD might be activated under these conditions.

It has recently been suggested, on the basis of PdtOH accumulation and inhibition of AAG formation by propanolol, an inhibitor of phosphatidate phosphohydrolase, that DRG were formed by sequential action of PLD and PdtOH phosphohydrolase in FMLP-stimulated neutrophils pretreated with cytochalasin B (31). Moreover, the observed accumulation of either alkyl-PdtOH and alkyl-DRG provided additional evidence that PdtOH and DRG are generated by hydrolysis of phosphatidylcholine by a PLD (22, 31). We have found in GM-CSF-treated neutrophils that 100 μM propanolol increased the basal level of PdtOH from 0.109 ± 0.026 to 0.244 ± 0.07 $\mu g/10^7$ neutrophils. In GM-CSF-treated neutrophils stimulated with FMLP for 2.5 min, propanolol increased the levels of PdtOH from 0.388 ± 0.054 to 0.483 ± 0.058 $\mu g/10^7$ cells. These effects of propanolol provide evidence for the presence of an active phosphatidate phosphohydrolase, and indicate that part of the DRG accumulation in GM-CSF-treated neutrophils stimulated with FMLP may derive from PdtOH via a phosphohydrolase. However, our results do not exclude DRG formation by a phospholipase C–mediated hydrolysis of phosphatidylcholine (40, 41).

Stimulation by FMLP of 1-0-[^{3}H]alkyl-2acyl-sn-glycero-3-phosphocholine-prelabeled neutrophils, led to rapid and enhanced formation of [^{3}H]alkyl-PdtOH in GM-CSF-pretreated neutrophils. In addition, in the presence of ethanol, an increased formation of [^{3}H]alkyl-PEt (concomitant with a decreased generation of [^{3}H]alkyl-PdtOH) was observed in GM-CSF-pretreated neutrophils challenged with FMLP. As PLD catalyzes *trans*-phosphatidylation in presence of alcohols (21), we concluded that GM-CSF upregulates a phosphatidylcholine-specific PLD in FMLP-stimulated neutrophils.

The RP-HPLC analysis of naphthylurethane derivative of neutrophil DRG, in addition to providing quantitative data, also unravelled specific alterations in the DRG profiles of neutrophils under various experimental conditions. Identification of the various DRG species, based on coelution with authentic standards is tentative and requires confirmation. Preliminary mass spectrometric analysis (data not shown) already confirmed that neutrophil DRG contain all the species detected by HPLC (and several others) and, furthermore, confirmed the major

change observed in DRG species after FMLP stimulation of GM-CSF-treated neutrophils (Fig. 2), i.e., an increase mainly in $C_{18:0}/C_{20:4}$ AAG. Studies are currently in progress to characterize DRG and PdtOH profiles in resting and activated neutrophils. Such analysis of DRG and PdtOH species are crucial to the understanding of the regulation of the pools of precursors of bioactive lipids, such as arachidonic acid (44–46), and of cellular responses, since AAG and EAG species have different regulatory properties on PKC activity and superoxide production (47).

It is noteworthy that there are several similarities between the effects of GM-CSF and the fungal metabolite cytochalasin B. We have found that neutrophil pretreatment with cytochalasin B or GM-CSF similarly enhanced the accumulation of DRG upon stimulation with FMLP (see also reference 31), and that cytochalasin B treatment of GM-CSF primed neutrophils did not cause a cumulative increase of DRG (data not shown). Like GM-CSF, cytochalasin B primes neutrophils for enhanced functional responses, including degranulation (48), respiratory burst (35), and phospholipase A_2 activation (46). Our results clearly suggest that the priming effect of GM-CSF on the respiratory burst in FMLP-stimulated cells is due to the enhanced sustained formation of DRG in these cells and the possible subsequent activation of the PKC (49). DRG and PdtOH, through DRG lipase (44) or specific PdtOH phospholipase A_2 (45), are both potential sources for the enhanced release of free arachidonic acid observed upon treatment of cells with GM-CSF (10).

The data presented in this paper demonstrate that the comprehensive description of the mechanism of action of GM-CSF will have to include the upmodulation of PLD activity. Increase of FMLP cell surface receptors (6) or enhanced expression or function of a possible GTP regulatory protein (7, 17) may play a role in the upregulation of PLD in neutrophils pretreated with GM-CSF. Alternatively, the increase in pHi induced by GM-CSF (7) may regulate PLD activity. Indeed, evidence has been presented suggesting that intracellular alkalinization secondary to Na^+/H^+ exchange regulates hormone-induced sustained diacylglycerol accumulation (50). Finally, tyrosine phosphorylation (7) or changes in cyclic nucleotide levels (19) induced by GM-CSF may modulate PLD activity.

The physiological consequences of the stimulated generation of PdtOH remain to be examined. PdtOH has been shown in a variety of cellular systems to possess growth factor–like activity (51–54), and to activate phosphoinositide hydrolysis (51, 55). Further experiments are thus needed to examine the potential (direct or indirect) roles that PdtOH may play at the levels of calcium mobilization and superoxide production, two functions primed by GM-CSF treatment of human neutrophils (6, 8).

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