The Regulation of Phospholipase-A2 (PLA-2) by Cytokines Expressing Hematopoietic Growth–Stimulating Properties (44006)

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Abstract. **Various growth factors released by macrophages and other cell types modulate normal hematopoiesis. The physiological mechanisms whereby these molecules interact with specific target cells are ill defined. Eicosanoids, the products of fatty acid metabolism, are known to regulate cell proliferation and differentiation. The release of membrane-bound phospholipid by phospholipase-A2 (PLA-2) is the first critical step in the initiation of membrane remodeling and eventually eicosanoid synthesis. We report here data that demonstrates how various cytokines exhibit a marked hydrolytic activity mediated through PLA-2 against both [l-^{14}C] oleic acid– and [l-^{14}C] arachidonic acid–labeled *Escherichia coli* (micelle) substrates. PLA-2 extracts were prepared from neutrophils elicited by injecting rats ip with 8% glycogen. The rate of hydrolysis of free fatty acids from the phospholipid substrate was found to be linear, rapid, and pH dependent and was calculated to be 30 nmoles of phospholipid/hr/mg protein lysate. Cytokines (i.e., interleukin-1 [IL-1, human and murine recombinant, α], mouse lung cell–derived colony-stimulating factor [L-CSF], granulocyte-macrophage colony–stimulating factor [murine recombinant GM-CSF], tumor necrosis factor [murine recombinant TNF-α], and granulocyte colony–stimulating factor [human recombinant, G-CSF] all induced PLA-2 activity with the release of free fatty acids above basal levels. In contrast, lipopolysaccharide (LPS), interleukin-2, (IL-2, human recombinant), and macrophage colony–stimulating factor (M-CSF) did not significantly activate PLA-2 hydrolysis. The activation of this membrane-bound enzyme-substrate complex by these growth factors may serve as a mechanism whereby the appropriate target cells expressing receptors respond through either direct or secondary signals leading to the formation of free fatty acids with the eventual synthesis of prostanoid or lipoxygenase products, resulting in cellular proliferation and differentiation.**

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An interrelated series of growth factors modulate the biochemical activity of target cells (1, 2). These growth factors are essential for *in vitro* clonal growth and maturation of hematopoietic progenitors. The precise mechanism of action by these factors on target cell activity is still ill defined. Growth factors such as granulocyte-macrophage colony–stimulating factor (GM-CSF), granulocyte colony–stimulating factor (G-CSF), macrophage colony–stimulating factor (M-CSF), tumor necrosis factor–α

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(TNF-α), and interleukin-1 (IL-1) not only affect cell growth but also modulate target cell activity (3, 4). Human recombinant GM-CSF stimulates the proliferation and differentiation of myelomonocytic stem cells *in vitro* (5). G-CSF and M-CSF appear to influence the determined final pathway that uncommitted hematopoietic progenitors will proceed either towards myeloid or monocytic differentiation respectively. TNF-α and IL-1 indirectly stimulate a variety of other cell types which produce and release other hematopoietic CSFs (6, 7).

The mechanism of CSFs in their regulation and interaction with specific target cell receptor(s) is just beginning to be elucidated in experimental hematology. Such interactions have been implicated to involve a multitude of varied responses. For instance GM-CSF "primes" neutrophils to enhance the pharmacological response to calcium ionophore and leukotriene B_4 production. G-CSF and TNF-α affect cell growth and influence neutrophils towards an enhanced responsiveness to a number of external influences (8, 9). At the pharmacological level, neutrophil/monocyte stimulation involves a multitude of processes, including an increase Na^+/H^+ antiport system, Ca^{+2} fluxes, NADPH oxidase activity and stimulation of membrane-associated and cytosol-mediated phospholipase activity (8–12). GM-CSF and IL-1 have been shown to enhance both neutrophils and fibroblasts by releasing free fatty acid arachidonic acid (13, 14). Once free fatty acid is released via phospholipase (as a acylation/deacylation step) activity, metabolism of either membrane-bound cyclooxygenase and/or cytosolic 5-lipoxygenase pathways can be activated. Prostaglandins modulate erythroid differentiation, whereas lipoxygenase products influence the signal induction on target cells by GM-CSF (15).

In this investigation, we report evidence to suggest that several growth factors, all expressing hematopoietic activity, stimulate the activation of a membrane-bound, phospholipase-A2 (PLA-2). This enzyme catalyzes the formation of both prostaglandins and lipoxygenase products, and is considered the first rate-limiting step in the level of free arachidonate made available to cells which are used in the synthesis of their ecosanoid products which ultimately influences their response to activation.

Materials and Methods

Growth Factors and Reagents. Purified recombinant human GM-CSF (specific activity: 10^8 units/mg protein) expressed in bovine papilloma systems was a gift from Dr. Anthony Beck (Sandoz Pharmaceuticals, Geneva, Switzerland). Recombinant human IL-1 (specific activity: 1.9×10^7 units/ml) and IL-1 antisera were kindly supplied by Dr. Peter Lomedico (Hoffman-LaRoche, Nutley, NJ). Mouse L-CSF (a conditioned medium, specific activity: 10^3 units/mg protein) and L-CSF antisera were prepared as described (5) was a generous gift from Dr. Richard E. Stanley (Albert Einstein College of Medicine, Bronx, NY). IL-2 was obtained from Cetus (Emeryville, CA) (specific activity: 10^6 units/mg protein). M-CSF (specific activity: 10^8 units/mg protein) and G-CSF (specific activity: 10^6 units/mg protein) were a kind gift from Dr. Francis Ruscetti (Laboratory of Molecular Immunoregulation, Cancer Research Center, Frederick, MD). Murine recombinant TNF-α (specific activity: 2.9×10^7 units/mg) and polyclonal rabbit antisera to murine TNF-α were supplied by Genetech, Inc., (San Francisco, CA). Macrocortin and the monoclonal antibody to macrocortin (Rm 233) was generous gift from Dr. Rodney J. Flower, School of Pharmacy and Pharmacology, University of Bath, United Kingdom; quinacrine hydrochloride and mellitin were purchased from Sigma Chemical Co., (St. Louis, MO). GTP-α S, and GDP-β S were purchased from Boehringer Mannheim (Indianapolis, IN).

Neutrophil Cell Preparation. Preparation of purified neutrophils was conducted using rats (Wistar, 8- to 10-week-old males, Charles River, Fall River, MA). Animals received an intraperitoneal injection of 8% glycogen (Sigma) in a 10-ml volume. After 24 hr, rats were sacrificed by CO_2 asphyxiation. Neutrophils were harvested by peritoneal lavage and then isolated by a one-step Ficoll method by using resolving media (Flow laboratories) (16). Any contaminating red cells were removed by hypotonic lysis. This procedure yielded a population of cells that were 98% neutrophils based on morphological examination by Wright/Giemsa staining.

Preparation of PLA-2 Extracts. Membrane-associated PLA-2 was isolated and solubilized as described (17, 18). In short, the peritoneal exudates were harvested along with 3.0 ml of phosphate-buffered saline (PBS). The cell suspensions containing 3×10^8 cells were pooled and centrifuged to pellet the cells. After removal of the supernatant fluid, 200 μL of cold 0.15 *N* sulfuric acid was added, and the pellet was homogenized with a Potter-Elvehjem homogenizer. The homogenate was left for 45 min at 4°C, homogenized again and centrifuged for 20 min at 15,000 *g*. Again the pellet was resuspended in 8 ml of Tris-HCL Buffer (100 m*M*), 5 m*M* $CaCL_2$, pH 7.5. The amount of protein in the resulting supernatant fluid enriched in PLA-2 activity was determined by standard procedures and dialyzed extensively against acetate buffer (5 m*M*, pH 5.0). This suspension was then filtered through nylon gauze (mesh size: 900 μm) and then centrifuged at 450 *g* for 10 min at 4°C. The pellet contained whole cells, nuclei, and other cellular organelle components. The supernatant was then recentrifuged at 15,000 *g* for 25 min at 4°C. The plasma membrane

pellet was carefully resuspended in a 100 m*M* Tris buffer and layered on top of a 35% (weight by weight) sucrose solution and recentrifuged at 24,000 *g* for 50 min at 4°C. The plasma membrane enriched fraction, containing the PLA-2 enzyme, was collected at the sucrose-Tris interface and was carefully removed with a Pasteur pipette and resuspended in a particular volume of 100 m*M* Tris buffer, 5 m*M* $CaCl_2$, (pH 7.5) and dialyzed against an acetate buffer, 5 m*M* (pH 5.0) twice. The membrane enriched preparation was subsequently collected by centrifugation at 100,000 *g* for 17 min at 4°C. Finally the amount of protein was determined by Lowry method (18) and subsequently used in the PLA-2 enzyme system or stored at −20°C and assayed within a 1-week period.

Permeabilization. The whole neutrophil uniformly labeled previously, with ^{14}C arachidonic acid–labeled phosphatidyl 1-choline in the 2 position for 15 min at 37°C, and/or the resulting cell lysate preparation to allow quanine nucleotides to enter the cell was accomplished via the technique of Burch *et al.* (19). Briefly, 3.7×10^8 neutrophils were incubated by plating cells in 12-well plates without fetal calf serum but in the presence of saponin at a concentration of 15 μg/ml for 5 min. In addition, the media contained quanosine-5-(γ-thiol)triphosphate (GTP-γ S) or guanosine-5-(β-thiol)diphosphate (GDP-β S). Subsequently, the wells were rinsed three times with culture media without saponin, but containing respective growth factors or controls. Then the cells were incubated at 37°C for 60 min. The reaction mixture was then stopped with the addition of three volumes of $CHCl_3/CH_3OH$ (1:2, v/v) and vortexed twice. The samples were then placed in a shaking water bath at 37°C for an additional 10 min. The $CHCl_3$ was removed and evaporated under N_2. The dried residue was then redissolved in 100 μl of acetone and developed using thin-layer chromatography, along with reference materials (free-fatty acids, prostanoid, and lipoxygenase products run as controls) looking for the synthesis of free fatty acids or prostanoid products.

Arachidonic Acid Incorporation and Release. Granulocytes were suspended at 3.7×10^8 cells/ml in calcium- and magnesium-free modified hanks' balanced salt solution (HBSS). An 0.01-ml aliquot of [^3H]-oleooyl- or 2-[^3H]-arachonyl-phosphatidylcholine (100 μCi/ml, Amersham Corp., Arlington Heights, IL) was added to the 1 ml cell suspension and incubated for 15 min at 37°C. The use of this label has been previously demonstrated by Nitts *et al.* (20), which assures the positional specificity of PLA-2 activity which was assessed by the formation of intracellular radioactive oleic acid or arachidonic acid. After the incubation with the appropriate reagents, total lipids were extracted accordingly to the method of Bligh and Dyer (21). The phases were separated by the addition of 0.5 ml of chloroform and 0.5 ml of 0.2 *M* KCl 0.5 m*M* EDTA mixture. After centrifugation at 1000 *g* for 10 min the lower organic phase was removed and evaporated under a stream of nitrogen gas. Lipids were dissolved in 50 μl chloroform/methanol (6:1, v/v). Neutral lipids were separated on Silica Gel 60 plates impregnated with 0.4 *M* boric acid, using chloroform/acetone (24:1, v/v) in a solvent system (22). Phospholipid separation involved 2-dimensional TLC (Silica Gel 60 plates impregnated with 2.5% magnesium acetate) was performed with chloroform/methanol NH_4OH (65:35:6, v/v) as solvent system for the first dimension, and chloroform/acetone/methanol/acetic acid/water (3:4:1:1:0.5, v/v) in the second dimension (20). Lipids were visualized by exposure of the plates to iodine vapor and identified by comigration with authentic standards.

Measurement of PLA-2 Activity. PLA-2 activity was measured by the hydrolysis of [I-^{14}C] arachidonic acid–labeled autoclaved *Escherichia coli* substrate (prepared by Dr. Richard C. Franson, Department of Biochemistry, Virginia Commonwealth University, Richmond, VA) (equivalent to 10 nmoles of phospholipid). Briefly, neutrophil plasma membrane extract (concentration of protein previously determined) was incubated with an appropriate concentration of growth factors or inhibitors. The concentration of each was previously established by values that optimally influenced the growth of hematopoietic progenitors cultured in semisolid media as performed routinely in the laboratory. The reaction mixture was carried out in a shaking water bath at 37°C for 60 min. The reaction was stopped by the addition of three volumes of $CHCl_3/CH_3OH$ (1:2, v/v) and vortexed twice. Samples were then placed in a shaking water bath at 37°C for an additional 10 min. The $CHCl_3$ phase was removed and evaporated under N_2. The dried residue was redissolved in 100 μl of acetone, spotted on aluminum backed thin-layer chromatographic plates, and developed in a solvent system consisting of petroleum ether-diethylether acetic acid (80:20:10). The lipids were visualized by exposure of I_2 vapor and the radioactive spots corresponding to standard phospholipid and/or free fatty acids were but from the plate. Radioactivity on the LC strips was determined by the addition of 1 ml of CH_3OH and 9 ml of hydroflur and counted in a liquid scintillation counter. Percentage hydrolysis was determined using the following equation:

$$\frac{\text{Free Fatty Acid (cpm)}}{\text{Total Phospholipid and Free Fatty Acid}}.$$

Rate of hydrolysis (nmoles/minute) was calculated as follows:

$$\frac{\%\ \text{Hydrolysis} \times \text{Phospholipid (10 nmoles)}}{\text{Incubation Time (min)}}.$$

Results

We attempted in these studies to demonstrate how various growth factors can induce both the stimulation and release of free fatty acids by the activation of an acid extracted neutrophil membrane-bound PLA-2. This is proposed to be an important first step in the growth factor induction of transmembrane signaling on target cells.

The following parameters have been established in this assay system. The acid-extracted neutrophil-rich membranes were the source of the PLA-2 enzyme, with a final concentration of 275 μg/test, and the *E. coli* micelle was the substrate labeled with either [I-^{14}C] oleic or [I-^{14}C] arachidonic acid (Fig. 1). The rate of hydrolysis of free fatty acids from the phospholipid substrate complex was found to be linear, rapid, and pH dependent (Fig. 2 and 3). The rate of hydrolysis under these optimum conditions was calculated to be 30 nmoles of phospholipid/hr/mg of protein lysate. Once the assay system was established, it was tested. A natural stimulator of PLA-2 is mellitin, which is a protein constituent extracted from bee venom previously shown to stimulate PLA-2 directly (23). In our system, mellitin was titrated against both a fixed concentration of enzyme substrate and fatty acid *E. coli* complex, and the results of these studies illustrate the system was functional with an optimum release of free fatty acids seen at 60 p*M*. Agents know to inhibit PLA-2 activity were also tested in this assay system. Quinacrine, an acridine derivative which irreversibly binds to sites either on the enzyme or substrate components of this system, inhibits PLA-2 background and stimulated the release of free fatty acid (FFA) by 90% with the IC_{50} at 40 p*M*. Macrocortin, a naturally occurring protein of molecular weight of 18,000 kDa specifically induced by glucocorticoid treatment of either monocytes/macrophages (24), inhibited background FFA release by 72% at an IC_{50} of 120 p*M*, but stimulated release was only attentuated by 32%. Macrocortin inhibition observed at the stimulated level was overcome by the addition of a higher concentration of the growth factor stimulant. To demonstrate the specificity of macrocortin activity, a monoclonal antibody specific for macrocortin, Rm 233, was employed and was shown to neutralize the inhibition observed by macrocortin in this assay system.

It has previously been demonstrated that calcium ions are necessary for the maintenance of full activity in this enzyme system (25). Verapamil, a calcium channel blocker, was tested both in the cell-free system (acid extract PLA-2) and the intact neutrophil.

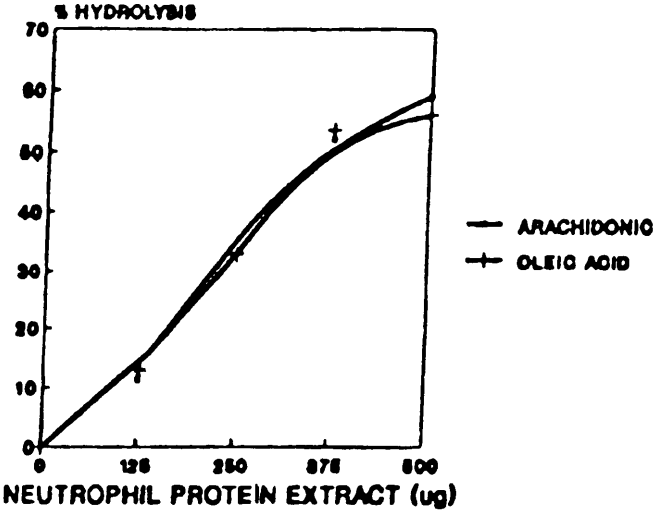

Figure 1. The rate of hydrolysis demonstrating both the release of free oleic and arachidonic acid from *E. coli* substrates, and linear release of phospholipids up to 375 μg of protein lysate. Percentage hydrolysis equals free fatty acid (dnp) divided by total phospholipid plus free fatty acids (dnp).

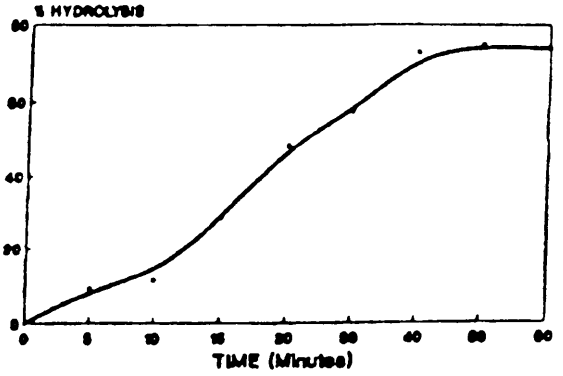

Figure 2. The rate of hydrolysis with respect to time and a fixed protein concentration of neutrophil lysate (275 μg) protein. Reaction rate was both rapid and linear up to 40 min. Percentage hydrolysis equals free fatty acid (dnp) divided by total phospholipids plus free fatty acids (dnp).

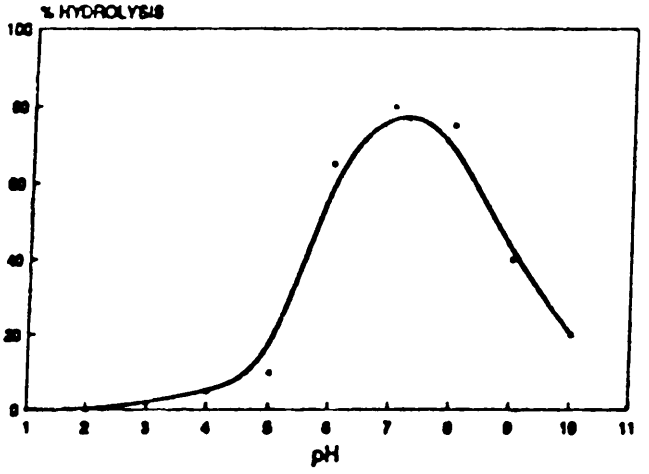

Figure 3. The rate of hydrolysis with respect to pH. The maximum activity for the hydrolysis of free fatty acid was 82% at a pH of 7.0. Percentage hydrolysis equals free fatty acid (dnp) divided by total phospholipids plus free fatty acids (dnp).

Figure 4 illustrates how the addition of verapamil to the cell free system inhibited the hydrolysis of FFA by 48% with an IC_{50} of 100 p*M*. The inhibition due to verapamil is most likely the result of its displacement of calcium ions on the enzyme component, which alters its binding conformation to the *E. coli* substrate complex (26). To demonstrate verapamil's true calcium channel–blocking activity, intact rat neutrophils were incubated with varapamil (0–100 μM). Verapamil attentuated the release of FFA over baseline levels and growth factor–induced release within an IC_{50} of 75 μ*M*. In addition, EGTA was employed to demonstrate how the chelation of external calcium ions at 1.5 m*M* concentration resulted in no release of FFA, which also demonstrates how important external calcium ions are for optimum enzyme activity.

To establish further that arachidonic acid release in mature neutrophils is a PLA-2–mediated event, experiments were performed with both cell-free membrane preparations and with whole neutrophils. Mature rat neutrophils were isolated as previously described and prelabeled with [$I^{14}C$] arachidonic acid–labeled phosphatidyl 1-choline in the 2 position for 15 min at 37°C (27). Then, either plasma membranes were prepared as previously described or the whole neutrophil was made permeable with a short treatment of saponin. GTP-γ S, a poorly hydrolyzed GTP analog that activates G proteins, was then titrated against a previously determined concentration of membrane protein (275 μg). Similar to the results seen in Figure 4, GTP-γ S at a 1-m*M* concentration was observed to attentuate the release of FFA (Fig. 5).

To determine if G proteins could possible be involved in the growth factor release of fatty acids, the growth factors previously tested in this study that demonstrate PLA-2 activity were combined with

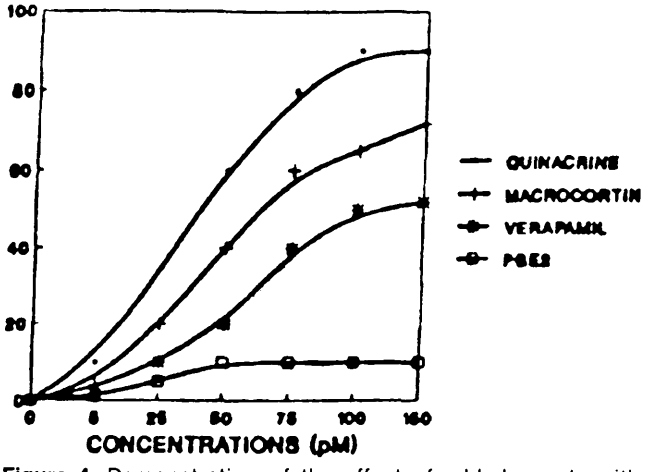

Figure 4. Demonstration of the effect of added agents with known phospholipase activity: quinicrine, macrocortin, verapamil, and PGE2. Percentage hydrolysis equals free fatty acid (dnp) divided by total phospholipids plus free fatty acids (dnp).

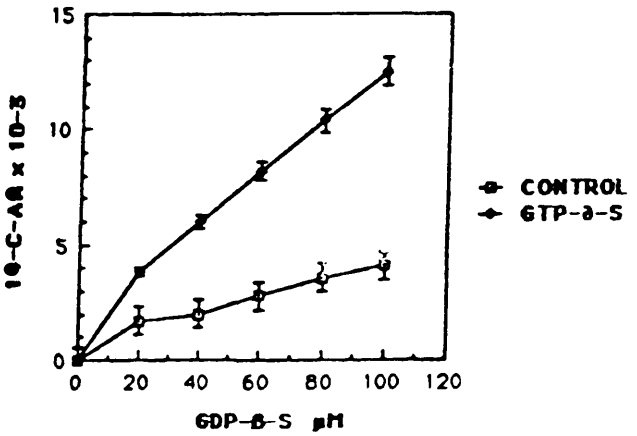

Figure 5. The rate of hydrolysis of [^{14}C] arachidonic acid in the presence of either GTP-γ S (1 m*M*) and increasing concentrations of GDP-β S ± SEM; *n* = 3. Percentage hydrolysis equals free fatty acid (dnp) divided by total phospholipids plus free fatty acids (dnp).

GTP-γ S at a 100-μ*M* concentration. In all instances in which growth factor + GTP-α S was tested, and enhanced release of FFA was observed following the growth factor + GTP-γ S combination versus either growth factor or GTP-γ S alone (Table I). Then GDP-β S was added to GTP-γ S or growth factor alone. In fact, the hydrolysis of FFA was reduced to within control levels. In addition, if a growth factor (i.e., IL-2) that previously did not demonstrate any degree of hydrolysis was combined with GTP-γ S no hydrolysis resulted over GTP-γ S alone. It appears that GTP-γ S is required for full growth factor activation of PLA-2 in the rat neutrophil. This demonstrates how essential it is for growth factor receptor–ligand complex to take place prior to or simultaneously with such activity for full PLA-2 activity to occur.

Since the mechanism of growth factor induction is still unknown, the scope of this study was to test whether growth factors could stimulate the release of FFA by the activation of a neutrophil plasma membrane-bound acid-extracted PLA-2. The results of these growth factor induced effects are listed in Table II. IL-1 is a protein growth factor with a molecular weight of 17.5 × 10^3 kDa. In addition, IL-1 has also been shown to be produced in several tissue types including macrophages, monocytes, fibroblasts, and neurons (28). FFA was released upon addition of IL-1 in our system with half maximal stimulation observed at 30 p*M* and maximal stimulation occurred by 60–70 p*M*. The colony-stimulating factors are a known family of glycoproteins which regulate the survival, proliferation, and differentiation of hematopoietic progenitors as well as the functional activities of mature cells (29–32). Murine L-cell CSF, a glycoprotein with a broad effect on macrophage-derived hematopoietic precursors, hydrolyzed FFA at a half-maximal stimu-

Table I. PLA-2 Activity: Phospholipids Hydrolyzed/min × 10^8 Cells

Unit/p*M*	IL-1 + GTP (N*M*)	GM-CSF-GTP (n*M*)	G-CSF + GTP (n*M*)	L-CSF + GSF (n*M*)	TNF + GTP (n*M*)	Mellitin + GTP (n*M*)
0	5 ± 0.25	3 ± 0.12	5 ± 0.10	3 ± 0.08	5 ± 0.09	4 ± 0.3
10	74 ± 0.54	24 ± 0.18	27 ± 0.18	25 ± 0.16	22 ± 0.40	8 ± 0.14
25	36 ± 0.17	57 ± 0.36	33 ± 0.21	38 ± 0.23	33 ± 0.35	40 ± 0.36
50	54 ± 0.83	67 ± 0.43	53 ± 0.76	49 ± 0.17	41 ± 0.40	57 ± 0.21
75	63 ± 0.09	88 ± 0.19	64 ± 0.55	63 ± 0.34	57 ± 0.35	60 ± 0.51
100	65 ± 0.37	99 ± 0.24	66 ± 0.27	66 ± 0.52	56 ± 0.45	71 ± 0.51

Note. Rat neutrophils were isolated as described in Materials and Methods. Next, isolated rat neutrophils were resuspended at 3.7 × 10^8 cells/ml in HBSS. Cells were prelabeld with [^{14}C] arachidonic acid–labeled phosphadidyll-choline in the 2 position for 15 min at 37°C. The unincorporated arachidonic acid was washed away, and the cells were resuspended along with a predetermined concentration of the growth factors and GTP-γ S. The mixture was incubated at 37°C for 60 min, at which time the reaction was stopped, the arachidonic acid extracted and determined according to the method of Bligh and Dyer (21).

Table II. PLA-2 Activity: Phospholipids Hydrolyzed/min × 10^8 Cells

Units/p*M*	IL-1 (n*M*)	IL-2 (n*M*)	IL-3 (n*M*)	GM-CSF (n*M*)	CSF-1 (n*M*)	G-CSF (n*M*)	M-CSF (n*M*)	TNF (n*M*)	LPS (n*M*)	Mellitin (n*M*)
0	3 ± 0.13	3 ± 0.02	3 ± 0.01	2 ± 0.03	3 ± 0.01	3 ± 0.13	2.5 ± 0.13	2 ± 0.1	2 ± 0.1	2 ± 0.13
10	18 ± 0.24	4 ± 0.08	3 ± 0.2	16 ± 0.11	16 ± 0.09	13 ± 0.23	3 ± 0.13	11 ± 0.3	2.5 ± 0.1	2 ± 0.32
25	28 ± 0.15	2.5 ± 0.05	3.5 ± 0.1	32 ± 0.14	23 ± 0.17	28 ± 0.35	4.5 ± 0.21	18 ± 0.17	2.5 ± 0.1	42 ± 0.24
50	42 ± 0.27	3.5 ± 0.05	3 ± 0.1	35 ± 0.21	40 ± 0.19	33 ± 0.24	8 ± 0.15	28 ± 0.24	2 ± 0.1	51 ± 0.35
75	52 ± 0.13	3 ± 0.08	3 ± 0.1	46 ± 0.24	42 ± 0.34	44 ± 3.2	10 ± 0.21	34 ± 0.3	2.5 ± 0.1	63 ± 0.40
100	54 ± 0.18	2.5 ± 0.02	3 ± 0.1	48 ± 0.31	44 ± 0.24	45 ± 0.21	10 ± 0.22	35 ± 0.25	2.5 ± 0.1	66 ± 0.35

Note. Rat neutrophils were isolated as described in Materials and Methods. Next, isolated rat neutrophils were resuspended at 3.7 × 10^8 cells/ml in HBSS. Cells were prelabeled with [^{14}-C] arachidonic acid labeled phosphadidyll-choline in the 2 position for 15 min at 37°C. The unincorporated arachidonic acid was washed away, and the cells were resuspended along with a predetermined concentration of the growth factors. The incubation mixture was incubated at 37°C for 60 min, at which time the reaction was stopped, and arachidonic acid extracted and determined according to the method of Bligh and Dyer (21).

lation of 30 p*M* and maximal stimulation at 50–60 p*M*. GM-CSF, another granulocyte-macrophage progenitor cell–inducing factor with a molecular weight of 23 × 10^3 kDa, produced a half-maximal response at 15 p*M* with a maximal response plateauing at 40–50 p*M*.

Two additional growth factor glycoprotein molecules known to be involved in mechanism of granulopoiesis were also examined: G-CSF, which produces neutrophilic colonies, and M-CSF, which influences the maturation of monocyte progenitors. M-CSF did not significantly induce an increase in FFA formation over basal levels; however, G-CSF did stimulate the release of FFA with a half-maximal response observed at 15 p*M* and a maximal stimulation between 40 and 50 p*M*. These findings help explain why the GM-CSF was observed to cause the release of FFA, since the G-CSF receptors are present on the neutrophil membrane.

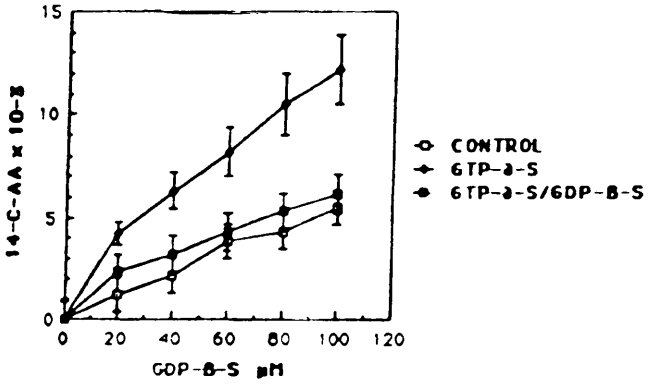

Figure 6. The rate of hydrolysis of [^{14}C] arachidonic acid in the presence of either GTP-γ S, GTP-γ S + GDP-β S, or increasing concentrations of GDP-β S ± SEM; *n* = 3. Percentage hydrolysis equals free fatty acid (dnp) divided by total phospholipids plus free fatty acids (dnp).

Tumor necrosis factor has emerged as a particularly important mediator of inflammatory responses (33). TNF-α has been demonstrated to stimulate the proliferation of normal fibroblasts and to activate polymorphonuclear leukocytes. In addition, TNF-α induces the release of certain growth factors including IL-1, GM-CSF, β_2-interferon and platelet-derived growth factor (34). TNF-α when introduced in our system released FFA with a half-maximal stimulation of 30 p*M* and a maximal stimulation between 40 and 50 p*M*. Another interleukin, IL-2, was also tested and found not to have any effect on the stimulation of PLA-2 activity in this system regardless of the concentrations tested. In addition, lipopolysaccharide (LPS), derived from gram-negative bacteria known to interact within the hematopoietic microenvironment and on specific target cell, did not produce any effect on the activation or conversion of substrate bound

phospholipids into FFA formation. In all instances when monoclonal antibodies were employed to prove specificity of each growth factor action in this system, neutralization resulted with no release in FFA observed.

Discussion

In this study an acid extraction from a neutrophil cell homogenate yielded a plasma membrane enriched with PLA-2 activity The rate of hydrolysis was directly proportional to the enzyme concentration and availability of the phospholipid substrate. The rate of FFA hydrolysis from both oleic and arachidonic acid was found to be linear, rapid, and pH dependent. It was noted that the acid extraction form of the plasma membrane enzyme components were extremely active even when compared with the PLA-2 activity found in either platelet homogenate or other commercial sources which are usually prepared under more neutral pH conditions. These results agree with those reported by both Chang *et al.* (35) and Kramer (36), who demonstrated that the use of an acid extracting procedure to prepare neutrophils significantly improved the recovery and possible conformational structure of the PLA-2 enzyme activity.

Growth factors have been generally known to exhibit innumerable effects on cells present in or constituting the hematopoietic microenvironment. Although many of these activities appear direct, such as activation, competence, and maturation of either myelomonocytic or erythoid precursors, many growth factor–induced responses seem to be indirect and require a secondary signal (37, 38). Examples of such growth factors include: GM-CSF, priming neutrophils for enhanced chemotactic and superoxide generation in response to chemotactic factors (39), and both IL-1 and TNF, conferring significant protection of hematopoietic progenitors by indirectly increasing levels of cAMP/PGE_2 and decreasing the myelotoxicity associated with either drug or radiation exposure (40). Once the capability of measuring the release of FFA formation was established, it was then possible to test the capacity of the growth factors known to have hematopoietic activity to influence PLA-2 formation (41). Mellitin, a known stimulator of PLA-2 activity, hydrolyzed the release of FFA in this system, while quinacrine, a known inhibitor of PLA-2 activity, significantly reduced the formation of PLA-2 in our system (Fig. 6). The relationship between these PLA-2 modulatory substances and their ability to influence hematopoietic progenitor colony formation has only recently been speculated and in fact preliminary data from our laboratory and results reported by Beckman *et al.* (42), have indicated that quinacrine, in the presence of optimal concentration of GM-CSF and erythropoietin, attentuates the formation of both granulocytic and erythroid progenitors.

It has been speculated that calcium ion flux influences the action of growth factors. Calcium channel blockers and chelating agents are useful agents that effectively alter the position of calcium both in isolated cell-free systems and influx in cells. Varapamil, a known calcium channel blocker, has been demonstrated to inhibit the synthesis of lipid mediators such as prostaglandin formation from endothelial cells (43). Because of this known relationship between calcium and lipid metabolism, it could be argued that varapamil may act by blocking an early step in metabolism of FFA. When the neutrophil-PLA-2-acid membrane extract was combined with varapamil at various concentrations along with *E. coli*–labeled substrate, inhibition of FFA hydrolysis resulted. In addition, when verapamil was introduced to intact rat neutrophil, attentuation in the release of both baseline and growth-induced FFA was observed at an IC_{50} of 75 μM. These findings agree in part with those of Chang *et al.* (35) utilizing both platelet-PLA-2 membrane extract and the intact mouse peritoneal macrophage. Obviously, other calcium antagonists must be examined and tested to evaluate whether structurally different calcium antagonists act in the same fashion. A functionally similar naturally occurring inhibitory molecule has been identified and isolated from parallel perfused lung (42) and from rat peritoneal lavage cells (44). This protein, which is specifically induced by glucocortocoids, has a molecular weight of 15,000 kDa and has been identified to be macrocortin (45). When macrocortin was introduced into this system, inhibition of FFA resulted by 72% with IC_{50} of 120 pM. When mellitin was introduced along with macrocortin in the system, only a 32% inhibition was observed. The extact mechanism of macrocortin inhibition is not known; however, the results from these studies speculate that macrocortin inactivates membrane-bound PLA-2 by interacting with the enzyme component from the outside of the cell, due solely to macrocortin's rapid degree of inhibition once added to the system. To demonstrate macrocortin's effect in this enzyme system, a monoclonal antibody to macrocortin, Rm 233, was employed. In the presence of added Rm 233 and macrocortin in the reaction mixture, a release of FFA was measured. Thus, the monoclonal antibody neutralized macrocortin's effect in this system.

Prostaglandins and related arachidonic acid metabolites have been shown to have a wide variety of pharmacological/biological effects on hematopoietic progenitors (46). Prostaglandin E_2 has been shown *in vitro* to inhibit myeloid colony development and augment erythroid growth (47, 48). Since prostaglandin E_2 appears to influence progenitor cell development, it

was added to our enzyme system either in the presence of enzyme or substrate simultaneously, or alone, and was without any increase in FFA release over basal levels.

Hematopoietic growth factors are a family of glycoproteins which influence the activation, survival, proliferation, and differentiation of hematopoietic progenitors. The signal transduction, negative or positive from outside the cell, which influences stem cell maturation is still an enigma. It was the purpose of this study to demonstrate how various growth factors and their receptors mediate signals which are linked primarily to PLA-2 and possibly other enzymes. IL-1, a prime mediator in the inflammatory response, has been demonstrated to have a wide variety of functions, some of which include inducting IL-2 by T cells and acting synergistically with GM-CSF to enhance myeloid progenitor colony growth (48). IL-1, either α or β, used in this enzyme assay system stimulated a significant release of FFA which was optimal at 65 p*M*. GM-CSF, another growth factor responsible for a multitude of effects on both immature cells at different stages of differentiation, when added to this membrane complex enzyme system did hydrolyze phospholipids to FFA *via* membrane enzyme PLA-2. Granulocytes possess receptors for GM-CSF, and this factor has been shown to enhance the expression of complement receptors and altered the affinity of FMLP receptors (49, 50). When mouse recombinant GM-CSF was introduced into this system, a half-maximal response of 15 p*M* was observed, with maximal response of FFA released between 40 and 50 p*M*. In the presence of human GM-CSF, no FFA was released, which verifies what has been observed in other systems comparing murine and human GM-CSF (50, 51). Murine L-cell CSF, a macrophage progenitor stimulatory molecule, has the capability of stimulating RNA synthesis in mature granulocytes and to binds to granulocytes with high affinity (52), stimulated the hydrolysis of FFA with maximum activity observed at 50–60 p*M*. This data is consistent with what has been observed throughout these studies, that growth factor receptors must be present on the surface of the rat neutrophil to stimulate the PLA-2 membrane enzyme system. Whether these growth factors were murine derived natural or in a recombinant form, further studies are required to confirm these findings using an acid-extracted PLA-2 from human neutrophils and other target cells as well as purified formed PLA-2.

These findings utilizing rat neutrophil acid-extracted plasma membrane preparations rich in PLA-2 activity support previous results observed with the intact neutrophil (53–58). More recent studies from our laboratory indicate that although growth factors stimulated PLA-2 from intact rat neutrophils, the amount of measurable breakdown products as a result of FFA liberation intracellularly was insufficient to quantitate using thin-layer chromatography (unpublished observations). Studies are presently in progress utilizing HPLC methodology to examine the full profile of arachidonate products. Preliminary data indicate that the breakdown products observed following growth factor stimulation are lipoxygenase products, since in the presence of the lipoxygenase inhibitor, nordihydroguauarettic acid and BW755C, the lipoxygenase products were no longer detected. These preliminary findings support and suggest that lipoxygenase products could act as intracellular second messengers for G protein gating ion channels (59). Whether the growth factors tested here act directly upon the PLA-2 alone or *via* a membrane receptor enzyme complex is currently under investigation.

TNF-α was first discovered as a protein component in the serum of endotoxin-treated animals (60). TNF-α has been shown to have both cytostatic and cytocidal activity against tumor cells *in vitro* (59). In addition, TNF-α has been shown to have both positive and negative effects on hematopoietic progenitor cells. TNF-α inhibits erythroid (BFU-E) and multipotential (CU-GEMM) progenitors (61). In contrast, TNF-α has also been shown to stimulate the production of GM-CSF from human lung fibroblasts and vascular endothelial cells (33). TNF-α has shown consistent activity in inducing polymorphonuclear neutrophil infiltration in the mouse peritoneal cavity (59–60). We do know that TNF-α acts directly on early progenitor cells; however, whether a secondary signal is involved or not is currently under investigation. When TNF-α was introduced into this test system, hydrolysis of FFA was seen with a half-maximal stimulation at 30 p*M* with a maximal stimulation at 45 p*M*.

Since there are other interleukins within this cytokine family, which promote hematopoiesis with broader effects, an additional family member, IL-2, was tested. When IL-2 was used in this enzyme system, no evidence of FFA release could be measured above basal levels. This finding is not surprising since no experimental evidence exists to demonstrate that IL-2 affects neutrophils biochemically or functionally. IL-3 has been demonstrated to possess progenitor stimulating activity (62). Generally, high-affinity receptors for IL-3 are not usually present on a wide variety of cell types; however, a number of cell lines which do become IL-3 dependent have receptors for IL-3 and upon isolation and preparation, plasma-membrane preparations act upon radioactive *E. coli* fatty acid substrate to cause the release of FFA (personal observation), but in this assay system IL-3 was shown not to have any effect. Observations by Lopez *et al.* (49) and Garland (63) have demonstrated that

IL-3 activates eosinophils but not mature neutrophils because membrane receptors for this growth factor are absent.

In this study, an attempt was made to demonstrate that various growth factors activate membrane-associated PLA-2 from the rat neutrophil. This enzyme system is believed to take an active part in the routine biology of phospholipid metabolism in biomembranes. Specific examples include eicosanoid biosynthesis and the acylation-deacylation of fatty acids. The growth factors tested do indeed activate PLA-2 from the neutrophil target cell, but by what mechanism and at what level enzyme substrate of guanine nucleotide regulatory binding protein are currently under investigation.

These experiments support earlier work which found that with TNF-α and the whole neutrophil, all growth factors that alone stimulated PLA-2 markedly increase the release of FFA when combined with GTP-γ S. In addition, when GTP-β S was substituted or combined with GTP-γ S, no marked increase of FFA could be measured (64). These observations suggest the involvement of G proteins, however, whether growth factors directly couple their receptors to a G protein or use some secondary mechanism is currently under investigation. In preliminary experiments testing whole neutrophils treated with both Pertussis and Cholera toxin upon acid-extracted membrane-bound PLA-2, results suggest a sensitivity to Pertussis toxin for all growth factors successfully tested in this system, and no effect with Cholera toxin treatment. This study attempted to demonstrate how various cytokines affect PLA-2 activity. It was a surprise to us that cytokine receptor presence appeared essential for hydrolytic breakdown and FFA formation. What we have not addressed in this study is whether each cytoline receptor(s) has a unique signaling pathway toward the activation of membrane-bound PLA-2, nor have we examined whether acid treatment affects cytokine receptor stability or activation. A common underlying thread, however, is the necessary equivalent for receptor presence and GTP for optimum hydrolytic activity when compared with neutrophil membrane preparations under more neutral pH conditions. This article focuses upon acylation/deacylation of FFA from membrane preparation or substrate-bound phospholipase. Subsequently, this important event leads to a multitude of effects, of which several were at least implicated in this study. In addition, we have tried to emphasize how essential calcium ions are for optimal release of FFA both in the intact cell and the isolated membrane or substrate bound enzyme. A theory we are currently testing and which previous data has suggested is that growth factors act in concert with calcium ions to decrease the binding activity of an "endogenous" growth factor inhibitor(s) (e.g., macrocortin) target cell. Growth factor initiation of PLA-2, with the release of FFA, as a critical first step in the mediation of G protein influence on target cells and their receptors is suggested by previous findings (65) and this study.

Furthermore, this study has suggested a novel approach to how growth factors may transduce their signal on their target cells. Whether growth factors directly stimulate PLA-2 or do so *via* membrane-bound receptors with the possible recruitment of G proteins has only been partially addressed. With the recent cloning, isolation, and purification of the membrane-bound form of PLA-2, it may be possible to confirm these findings, and this is currently being pursued. In addition, whether a direct or indirect interaction exists between the growth factor ligand-receptor complex and the PLA-2 enzyme system may also be brought to light. These findings strongly suggest that growth factors do cause the release of FFA, but the definite mechanism involved is still open to continued investigation of growth factor–modulated commitment towards stem cell proliferation and competence.

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