LYSOPHOSPHATIDES ENHANCE SUPEROXIDE RESPONSES OF STIMULATED HUMAN NEUTROPHILS[1]

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Abstract—Human neutrophils which are pretreated with subtoxic concentrations of a variety of lysophosphatides (lysophosphatidylcholine, lysophosphatidylcholine oleoyl, lysophosphatidylcholine myrioyl, lysophosphatidylcholine stearoyl, lysophosphatidylcholine gamma-*O*-hexadecyl, lysophosphatidylinositol, and lysophosphatidylglycerol) act synergistically with neutrophil agonists phorbol myristate acetate, immune complexes, poly-L-histidine, phytohemagglutinin, and *N*-formylmethionyl-leucyl-phenylalanine to cause enhanced generation of superoxide (O_2^-). None of the lyso compounds by themselves caused generation of O_2^-. The lyso compounds strongly bound to the neutrophils and could not be washed away. All of the lyso compounds that collaborated with agonists to stimulate O_2^- generation were hemolytic for human red blood cells. On the other hand, lyso compounds that were nonhemolytic for red blood cells (lysophosphatidylcholine caproate, lysophosphatidylcholine decanoyl, lysophosphatidylethanolamine, lysophosphatidylserine) failed to collaborate with agonists to generate synergistic amounts of O_2^-. However, in the presence of cytochalasin B, both lysophosphatidylethanolamine and lysophosphatidylserine also markedly enhanced O_2^- generation induced by immune complexes. O_2^- generation was also very markedly enhanced when substimulatory amounts of arachidonic acid or eicosapentanoic acid were added to PMNs in the presence of a variety of agonists. On the other hand, neither phospholipase C, streptolysin S (highly hemolytic), phospholipase A_2, phosphatidylcholine, nor phosphatidylcholine dipal-

mitoyl (all nonhemolytic) had the capacity to synergize with any of the agonists tested to generate enhanced amounts of O_2^-. The data suggest that in addition to long-chain fatty acids, only those lyso compounds that possess fatty acids with more than 10 carbons and that are also highly hemolytic can cause enhanced generation of O_2^- in stimulated PMNs.

INTRODUCTION

Recent studies from our laboratory (1–5) have described the role played by polycationic agents poly-L-arginine (PARG) and poly-L-histidine (PHSTD) as potent activators of the respiratory burst in human neutrophils (PMNs). It was also shown that a "cocktail" comprised of PARG, phytohemagglutinin, and cytochalasin B (CYB) caused generation of large amounts of superoxide (O_2^-) in human PMNs. Since a variety of cytolytic agents (lysophosphatidylcholine, digitonin, saponin) were able to replace PARG in the cocktail (5) and since PARG was also highly cytotoxic, we postulated that membrane-active agents including lysophosphatides might cause PMNs challenged with agonists known to activate the NADPH oxidase of PMNs (6, 7) to generate higher amounts of oxygen radicals. The present communication extends our earlier observations (5) and investigates in a systematic way the role played by lysophosphatides and certain fatty acids as potentiators of O_2^- generation in stimulated PMNs. The possible role played by lysophosphatides as amplifiers of the inflammatory response is discussed.

MATERIALS AND METHODS

Blood Neutrophils (PMNs).   Human blood in heparin (10 units/ml) was drawn from healthy donors. PMNs were isolated on a Ficoll-Hypaque gradient as described in detail elsewhere (4). Such preparations contained greater than 95% viable PMNs. Erythrocytes were removed by treatment with hypotonic saline followed by washing with normal saline buffered with 0.01 M phosphate, pH 7.3. The washed leukocytes were resuspended in Hanks' balanced salt solution (HBSS) buffered with 3 mM HEPES, pH 7.33, or in HBSS plus HEPES to which 10 mM sodium azide was added and kept on ice. Viability of the PMNs was evaluated by the trypan blue exclusion technique.

Measurement of Superoxide (O_2^-).   Superoxide was determined in stimulated PMNs by the reduction of cytochrome c (80 μM, type III, Sigma Chemical Company, St. Louis, Missouri) according to the method of Babior (7). The reaction mixtures contained $1-5 \times 10^6$/ml PMNs, and appropriate ligand (see below), cytochrome c, with or without 20 μg/ml superoxide dismutase (SOD) in a final volume of 1.0 ml. In some experiments, we examined the effect of CYB on O_2^- generation induced by the various ligands. The CYB was dissolved in dimethyl sulfoxide (DMSO)

at 500 μg/ml, and 2.5 μg/ml were employed. All reaction mixtures were incubated in a water bath at 37°C for various time intervals and then centrifuged at 1000g for 5 min. The optical density of supernatant fluids was read 550 nm. The amount of O_2^- was calculated from the extinction values using the formula $E_{550} = 2.1 \times 10^{-4}$ M and was expressed as nanomoles per given number of cells per 10 min.

Measurement of Hydrogen Peroxide (H_2O_2). Hydrogen peroxide was determined in stimulated PMNs by the method described by Thurman et al. (9). Briefly, to stimulated PMNs in HBSS plus NaN_3 (10 mM) (final volume of 1.0 ml), we added 200 μl of TCA (30%). The tubes were centrifuged at 1000g for 5 min. The clear supernates were transferred to clean tubes, and 200 μl of $Fe(NH_4)_2(SO_4) \cdot 6H_2O$ (19 mg/5 ml of water) plus 100 μl of KCNS (25%) were added. The tubes were agitated and incubated for 5 min at room temperature, and the brown color that developed was read in a spectrophotometer at 480 nm. A standard curve for hydrogen peroxide was prepared on the day of the experiment. The results were expressed as nanomoles per number of leukocytes per 10 min. Catalase (100 μg/ml, from bovine liver, 17,600 Sigma units/mg protein) was included in control tubes.

Stimulation of Oxygen Radical Generation. PMNs (1–3 $\times$ 10^6/ml) in HBSS containing sodium azide (10 mM) were treated for 15 min at 37°C with (A) PHSTD (molecular weight 15,000); (B) an immune complex (8) prepared by mixing 50 μl of a rabbit anti-bovine serum albumin (BSA) immune globulin containing 2 mg of protein nitrogen/ml, with 25 μg of BSA (6 mg/ml) (the resulting precipate was washed in saline and resuspended to the same volume); and (C) lipoteichoic immune complex [PMNs were pretreated for 10 min at 37°C with 25 μg/ml of lipoteichoic acid (LTA) derived from *Streptococcus pyogenes* (10); the PMNs were washed in HBSS and treated with a rabbit anti-LTA globulin containing 250 μg protein/ml in the presence of a cytochrome c]; (D) the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-5}–10^{-8} M); (E) phytohemagglutinin (PHA 50 μg/ml) derived from *Phaseoulus vulgaris*; (F) phorbol myristate acetate (PMA) (1–5 μg/ml); and (G) streptococci opsonized with rabbit anti-streptococcal serum and with complement (10).

Modulation of O_2^- Generation. The following agents were tested for their capacity to modulate the generation of O_2^- and H_2O_2: L-α-lysophosphatidylcholine from egg yolk, from bovine brain, and from bovine liver; L-α-lysophosphatidylcholine caproyl; L-α-lysophosphatidylcholine oleyl; L-α-lysophosphatidylcholine decanoyl; L-α-lysophosphatidylcholine myristoyl; L-α-lysophosphatidylcholine stearoyl; DL-α-lysophosphatidylcholine gamma-O-hexadecyl; L-α-lysophosphatidic acid oleyl sodium; L-α-lysophosphatidyl L-α-serine; lysophosphatidylethanolamine; L-α-lysophosphatidylinositol; L-α-lysophosphatidyl DL-glycerole; phosphatidylcholine; DL-α-phosphatidylcholine dipalmytoyl; palmitic acid; arachidonic acid; 5,8,11,15,17-eicosapentonic acid; phospholipase A_2 (from bee venom); phospholipase A_2 (from *Naja Naja*); phospholipase C (from *Clost. welchii*); and streptolysin S. All these agents were obtained from Sigma Chemical Company. The agents were either dissolved in saline or in ethanol followed by the addition of boiling water. All materials were kept at -20°C. Human PMNs (1–3 $\times$ 10^6/ml) in HBSS containing cytochrome c were pretreated for 1 min at room temperature with the various agents followed by the addition of a series of agonists (see above). In some experiments CYB (2.5 μg/ml) was included in the reaction mixtures. We also tested the superoxide-generating capacities of the various materials in the absence of added agonist.

Determination of Hemolytic Activity. One-milliliter aliquots of 1% suspension of human red blood cells in HBSS were treated for 15 min at 37°C with the various lipids or enzymes. The tubes were centrifuged at 1000g for 5 min, and the degree of hemolysis was assayed by measuring the absorption at 540 nm of the hemoglobin released. One hemolytic unit was determined to be as the smallest amount of agent that released 50% of the hemoglobin after 15 min of incubation.

RESULTS

Synergistic Effect of Lyso Compounds and Various Agonists in Superoxide Generation.   Since lyso compounds are generally known to be hemolytic for red blood cells and cytotoxic for nucleated cells, we first determined the ID_{50} for the various agents employing human red blood cells as targets. Table 1 shows that, except for lysophosphatidylcholine caproate, lysophosphatidylcholine decanoyl, lysophosphatidylethanolamine, and lysophosphatidylserine, all of the other lysocompounds tested were hemolytic for red blood cells at concentrations ranging from 5 to 10 μM. The various lyso compounds were tested for their capacity to enhance O_2^- generation in PMNs stimulated by various agonists using concentrations that induced less than 50% hemolysis of red blood

Table 1. Superoxide-Enhancing and Hemolytic Activities of Various Agents

Compound tested[a]	Molarity inducing	
	Hemolysis	O_2^- enhancement
Lysophosphatidylcholine (egg yolk)	8 μM	1-3 μM
Lysophosphatidylcholine (brain)	8 μM	1-4 μM
Lysophosphatidylcholine (liver)	8 μM	1-4 μM
Lysophosphatidylcholine caproate	None (10 mM)	None (10 mM)
Lysophosphatidylcholine decanoyl	None (10 mM)	None (10 mM)
Lysophosphatidylcholine oleyl	10 μM	3-5 μM
Lysophosphatidylcholine myristoyl	20 μM	5-7 μM
Lysophosphatidylstearoyl	6 μM	2.5-4.0 μM
Lysophosphatidylcholine-O-hexadecyl	15 μM	2-5 μM
Lysophosphatidic acid oleyl		
Lysophosphatidylethanolamine	None (10 mM)	None (10 mM)[b]
Lysophosphatidylserine	None (18 mM)	None (100 mM)[b]
Lysophosphatidylinositol	15 μM	5-12.5 μM
Lysophosphatidylglycerole	20 μM	1-2 μM
Arachidonic acid	None (200 μM)	30-50 μM
Eicosapentanoic acid	None (200 μM)	30-50 μM
Palmitic acid	500-700 μM	10-20 μM
Phospholipase A_2 (bee venom)	None (25 units/ml)	None
Phospholipase A_2 (*Naja naja*)	None (25 units/ml)	None
Phospholipase C	0.0125 units/ml	None
Streptolysin S	100 H units/ml	None

[a] PMNs were treated for 1 min at room temperature with the various compounds. The concentrations of the various agents employed to enhance O_2^- generation were below their hemolytic activity for a 1% suspension of human red blood cells. O_2^- generation was initiated either by BSA immune complex, poly-L-histidine, or PMA.

[b] A marked enhancement of O_2^- generation took place only in the presence of CYB (see Figure 6).

cells. Nonlytic compounds were tested at a wider range of concentrations. Figures 1–5 show the synergistic effects of lysophosphatides with a variety of agonists on O_2^- generation. All the lyso compounds that enhanced O_2^- generation also increased the production of hydrogen peroxide (not shown). None of the lyso compounds by themselves caused O_2^- generation when employed in the absence of neutrophil agonists. Table 1 also shows that all the other lyso compounds that hemolysed red blood cells also had the capacity to enhance O_2^- generation in stimulated PMNs. Of the lysophosphatides that failed to enhance O_2^- generation, two possessed fatty acids with less than 11 carbons (lysophosphatidylcholine caproate and lysophosphatidylcholine decanoyl). The other two nonactive compounds had a polar base (lysophosphatidylserine and lysophosphatidylethanolamine). Table 1 also shows that not all the agents that were hemolytically active enhanced O_2^- generation in stimulated PMNs. Specifically, neither phospholipase C nor streptolysin S affected the O_2^- response in neutrophils.

Since CYB is known to enhance O_2^- generation induced by a variety of both soluble and particulate agents (2, 11, 12), it was of interest to determine whether lyso compounds that were nonhemolytic and did not enhance O_2^- generation might do so in the presence of CYB. Figure 6 shows that in the presence
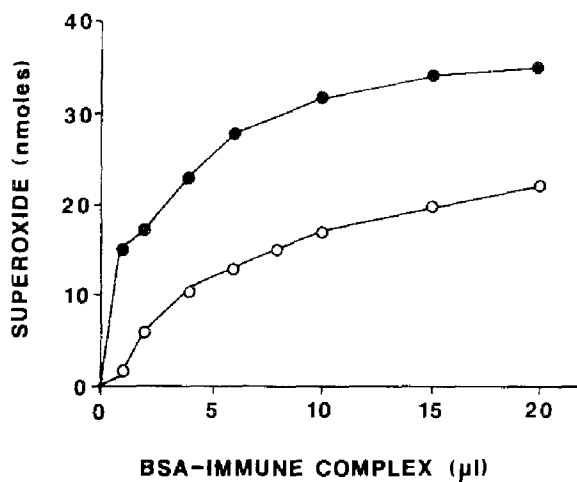

BSA–IMMUNE COMPLEX (µl)

Fig. 1. Effect of lysophosphatidylcholine (LL) on O_2^- generation induced by BSA immune complex. PMNs (2×10^6/ml) were treated with increasing amounts of BSA complex in the absence (O——O) and in the presence of LL (2.5 µg/ml ($\bullet$——$\bullet$). Note the marked enhancement of O_2^- generation in the presence of LL and that LL by itself did not generate O_2^- above the control levels.
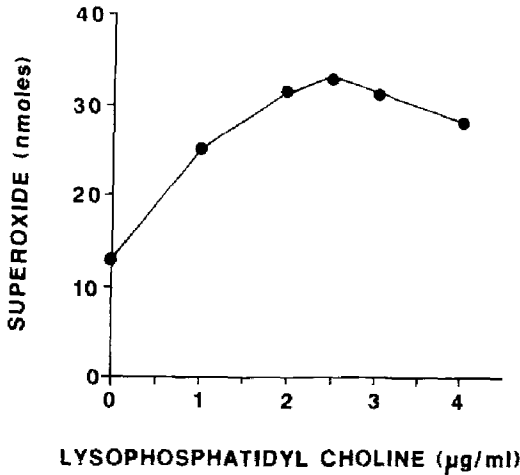
Fig. 2. Effect of lysophosphatidylcholine (LL) on O_2^- generation induced by lipoteichoic acid immune complex. PMNs (3×10^6/ml) were pretreated for 15 min with lipoteichoic acid (LTA). The cells were washed and resuspended in HBSS and further treated for 1 min at room temperature with increasing amounts of LL followed by the addition of anti-LTA globulin (250 μg protein/ml). Note that maximal stimulation of O_2^- generation ● —— ● took place with 2.5 μg/ml of LL, which is below its lytic capacity for red blood cells (see Table 1). LL by itself did not generate O_2^-.
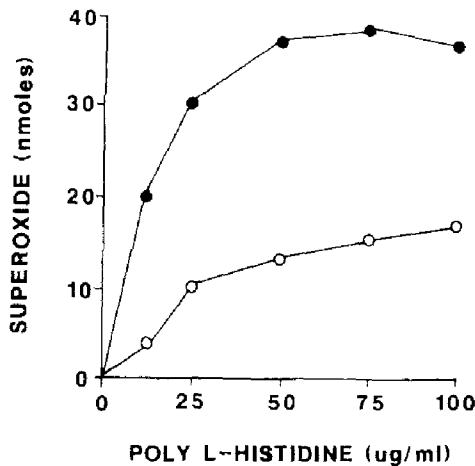

Fig. 3. Effect of lysophosphatidylcholine (LL) on O_2^- generation by poly-L-histidine (PHSTD). Untreated PMNs (2×10^6/ml) (○ —— ○) and PMNs pretreated for 1 min at room temperature with LL (2.5 μg/ml) (● —— ●) were challenged with increasing amounts of PHSTD. Note the marked enhancement of O_2^- generated by the combined effect of LL and PHSTD.
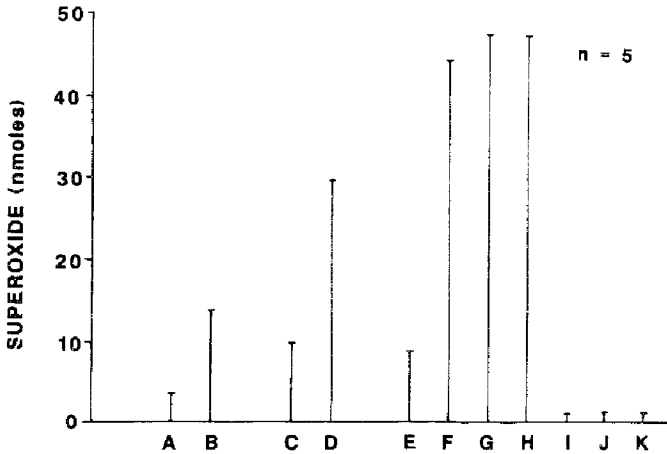
Fig. 4. Effect of lysophosphatides on O_2^- generation induced by various agonists. PMNs ($3 \times 10^6/$ ml) were treated with (A) FMLP (10^{-6} M), (B) FMLP + LL (2.5 μg/ml), (C) phytohemagglutinin (PHA) (50 μg/ml), (D) PHA + LL, (E) PMA (3 ng/ml), (F) PMA + LL, (G) PMA + lysophosphatidylglycerole (1.5 μg/ml), (H) PMA + lysophosphatidylinositol (2.5 μg/ml), (I) LL alone, (J) lysophosphatidylglycerol alone, and (K) lysophosphatidylinositol alone. Note the enhanced generation of O_2^- induced by the combination of lysophosphatides and various agonists.
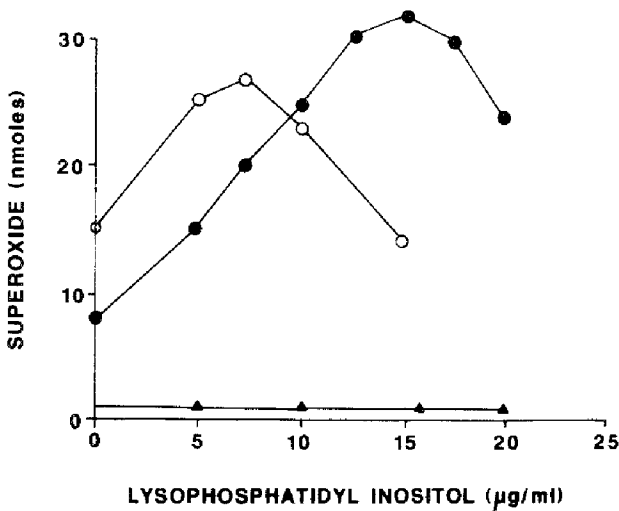
LYSOPHOSPHATIDYL INOSITOL (μg/ml)

Fig. 5. Effect of lysophosphatidylinositol (LPI) on O_2^- generation induced by two agonists. PMNs (2×10^6/ml) were pretreated for 1 min at room temperature with increasing amounts of LPI. The cells were then challenged either with poly-L-Histidine (PHSTD) (75 μg/ml) (O —— O) or with BSA immune complex (7.5 μl) ($\bullet$ —— $\bullet$). Note the distinct synergistic effects of the lysophosphatides with the two agonists on O_2^- generation. LPI alone ($\blacktriangle$ —— $\blacktriangle$) had no O_2^- generating ability.

of CYB even lysophosphatidylethanolamine and lysophosphatidylserine (not shown) were highly stimulatory to O_2^- generation when immune complexes or PHSTD were employed as agonists.

Since the stimulatory effects of the various lyso compounds might be related to the presence of free fatty acid contaminating the preparations and since fatty acids have been shown to trigger O_2^- generation by PMNs (13, 14), we tested the capacity of certain fatty acids to enhance O_2^- generation in stimulated PMNs. Figure 7 shows that both aracidonic acid and eicosapentanoic acid at 10 μM (nonhemolytic at concentrations up to 200 μM) (Table 1) also markedly enhanced O_2^- generation induced by PMA. Similar results (not shown) were also obtained when PHSTD or BSA immune complex were the agonists. Under similar conditions at concentrations up to 10 μM, palmitic acid was only slightly stimulatory when employed in conjunction with either PMA, PHSTD, or with
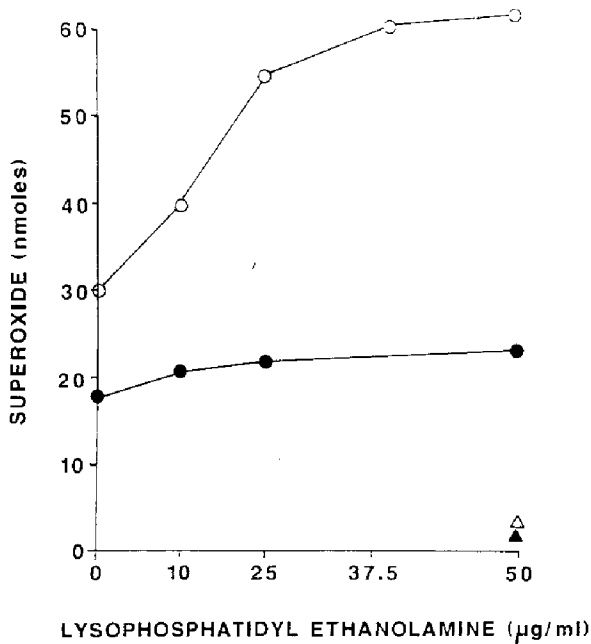

Fig. 6. Effect of CYB on O_2^- generation induced by lysophosphatidylethanolamine (LPEA) in the presence of BSA immune complex. PMNs (2 × 10⁶/ml) were pretreated for 1 min at room temperature with increasing amounts of LPEA. The cells were then further treated with BSA complex (7.5 μl) in the absence (●——●) and presence of CYB (2.5 μg/ml) (○——○). Note that while in the absence of CYB, LPEA failed to generate appreciable amounts of O_2^-, a distinct synergistic generation of O_2^- took place when PMNs were simultaneously treated with LPEA, BSA complex, and CYB. Neither LPEA (▲) nor LPEA + CYB (△) in the absence of added agonist had the capacity to generate O_2^-.
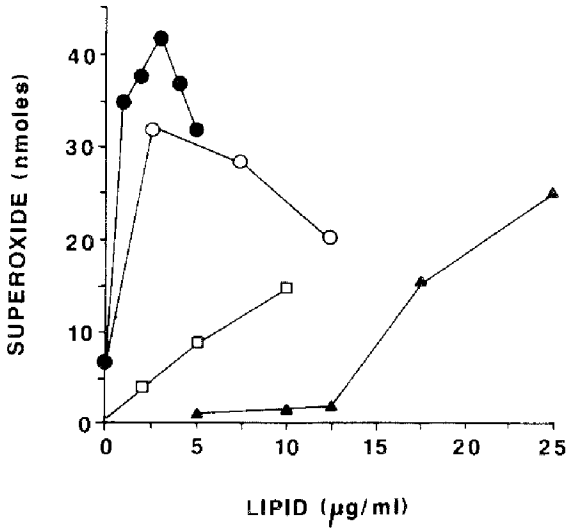
Fig. 7. Effect of free fatty acids on O_2^- generation induced by PMA. PMNs (2×10^6/ml) were treated with increasing amounts of arachidonic acid (AA) (▲——▲), eicosapentanoic acid (EICO) (□——□) with AA + PMA (33 ng/ml) (○——○) and with EICO + PMA (●——●). Note the distinct synergistic generation of O_2^- when fatty acids act in collaboration with PMA. The concentrations of the fatty acids that enhanced O_2^- generation in the presence of an agonist were far below those that induced O_2^- generation in its absence.

immune complexes (not shown). It should be noted that based on the dose-responses as compared to the amounts of lysophosphatidylcholine (Figure 1) required for enhancement of O_2^- responses in stimulated PMNs, it seems unlikely that the effect of the lysophosphatidyl compounds can be ascribed to presence of contaminating free fatty acids.

DISCUSSION

The data presented show that the generation of O_2^- by human PMNs is greatly enhanced in the cells that are pretreated with lysophosphatides and then challenged with a variety of agonists (Table 1, Figures 1–5).

The direct correlation between the hemolytic activity of the various lyso compounds and their capacity to synergize with agonists to generate enhanced amounts of O_2^- is striking (Table 1). Nonhemolytic compounds (lysophosphatidylcholine caproate, lysophosphatidylcholine decanoyl), that failed to hemolyse red blood cells also failed to act synergistically with neutrophil agonists. Since these compounds possess fatty acids with less than 11 carbons, it appears

that the length of the fatty acid chain might play an important role as a membrane permeabilizing agent. Furthermore, since both lysophosphatidylserine and lysophosphatidylethanolamine (which contain primarily stearic and palmitic acid) also failed either to hemolyse or to synergize with the agonists, we suggest that the presence of polar groups in these substances interfered with the permeabilization of the membrane or with the O_2^--generating system. Since the concentrations of the various lyso compounds that acted synergistically with the agonists were not cytotoxic, it appears that the various agents first interacted with some phospholipid targets in the PMNs membrane. This interaction "primed" the oxidase, which was then fully activated by a "second hit" (5, 6). Since none of the lysophosphatides employed in this study had the capacity to induce O_2^- generation in the absence of added agonist (see also 13), these compounds differ from digitonin (15) and from free fatty acids (14), which are efficient O_2^--generating agents. Quantitatively, however, much larger amounts of the free fatty acids are needed to generate comparable amounts of O_2^- (Figure 7). It seems, therefore, that the capacity of the various lyso compounds to synergize with the agonists is not due to contamination with free fatty acids.

Since two hemolytic agents (phospholipase C and streptolysin S) failed to synergize with the various agonists in O_2^- generation, it appears that not every membrane-active agent has the capacity to prime the oxidase in the membrane. The mechanism by which the various lytic agents augment O_2^- generation in the presence of other agonists is not fully known. The inability of phospholipase A_2 and phospholopase C, when added externally, to stimulate O_2^- generation is intriguing. Since activation of NADPH oxidase is known to involve the activation of the inositol phosphatide cascade (16–20) and the release of arachidonic acid from membrane phospholipids with the concomitant accumulation of lyso compounds, it appears that when added externally these phospholipases fail to gain access to their substrates, which are the products involved in the ultimate activation of the oxidase. This can be achieved, however, by adding the degradation product of phospholipase A_2, namely lysophosphatide. Further studies employing thin-layer chromatography might reveal whether or not the interaction of PLA_2 with the cells resulted in the accumulation of lyso compounds in the membrane.

More recently (21) it was also shown that lysophosphatidylcholine enhanced the generation of O_2^- induced by various soluble agonists. On the other hand, no such stimulation occurred when opsonized zymosan was the agonist. Our results vary from those described (21) in several aspects and add new observations. (1) Employing similar concentrations of the various lyso compounds, we found a direct correlation between the length of the fatty acids present in the various compounds and their hemolytic and O_2^--generating capacities (Table 1). (2) Although lysophosphatidylserine and lysophosphatidylethanolamine failed to enhance O_2^- generation when tested with the various

agonists (21), they did so in the presence of CYB (Figure 6). (3) The degree of stimulation obtained in our studies is much larger than that described (21) (Figures 1–5). (4) Not only lyso compounds, but also fatty acids such as arachidonic acid and eicosapentanoic acid, proved very effective primers of the oxidase when employed in collaboration with a variety of agonists (Figure 7). (5) The lyso compounds also markedly stimulated O_2^- generation by particulate agents such as opsonized streptococci (not shown) and by two immune complexes (Figures 1–3). Since it was found that nonmetabolizable analogs of platelet-activating factor were also active as stimulators of O_2^- generation in the presence of PMA (21), it is unlikely that the enhancement of O_2^- generation was due to the acetylation of the lyso compounds to generate platelet activating factor.

The significance of the synergistic effect of lysophosphatides with other agonists in the generation of large amounts of O_2^- might be important in sites of inflammation. Large amounts of lipid material are known to accumulate in inflamed sites. PLA_2 released from activated PMNs or macrophages might generate lyso compounds that further collaborate with other agonists, e.g., immune complexes, chemotactic peptide, etc., to generate large amounts of toxic oxygen species.

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