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PHAGOCYTOSIS-INDUCED RELEASE OF ARACHIDONIC ACID FROM HUMAN NEUTROPHILS

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Summary: The phospholipids of human neutrophils were labeled with [^{3}H] arachidonic acid and [^{14}C] palmitic acid. Phagocytosis of opsonized zymosan resulted in rapid release of free arachidonic acid but not of palmitic acid. Arachidonic acid was not released when the cells were exposed to unopsonized zymosan, zymosan-activated serum, or phorbol myristate acetate. These observations suggest that phagocytosis of opsonized zymosan results in the activation of a phospholipase A_2.

Introduction. Human polymorphonuclear leukocytes constitute a major line of defense for the host against the dangers of acute bacterial infection by virtue of their ability to phagocytize and kill the invading organisms. The cell first attaches to the bacteria and then surrounds the particle with pseudopods which meet and fuse, enclosing the particle within a membrane-limited vesicle, the phagosome, which is derived from the plasma membrane of the cell (1). Lysosomes, or more appropriately, granules, within the cytoplasm of the cell migrate to the phagosome and the membranes of the granules fuse with that of the phagosome so that the contents of the granules are now available to act upon the ingested particle (1). During this process, the cell also undergoes extensive alterations in metabolism which result in the production of toxic intermediates of oxygen including superoxide anion, hydrogen peroxide, and perhaps singlet oxygen and hydroxyl radical (2). These reactive intermediates are thought to be crucial to the bactericidal event and likely act in concert with the granule-bound enzymes to destroy the microorganism.

One might expect that the extensive membrane remodeling (fusion of pseudopodia and lysosome-phagosome fusion) would be reflected in alterations of lipid metabolism. Indeed, it has been known for some time that there is increased turnover of ^{32}P in phospholipid of the cell membrane (3), but other

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alterations in lipid metabolism have been difficult to document. A recent paper by Franson et al. (4) has reported the presence of a neutral-active phospholipase A_2 in the plasma membrane of human neutrophils. In the present communication we describe an increased release of free arachidonic acid from neutrophils phagocytizing opsonized zymosan; this may be necessary for the extensive membrane alterations which occur and is consistent with a phagocytosis-induced activation of a membrane-bound phospholipase.

Methods. Polymorphonuclear leukocytes were obtained from peripheral blood of normal healthy volunteers by a previously published procedure (5). Briefly, erythrocytes were sedimented with plasma gel for 45-60 minutes and the supernate centrifuged for 8 minutes at 250 xg. The leukocyte pellet was washed once with 5.0 ml Dulbecco's phosphate buffered saline (PBS) by suspension and recentrifugation. The few contaminating erythrocytes were lysed by a brief (10 seconds) exposure to distilled water and isotonicity was restored by the addition of an appropriate volume of 3.5% sodium chloride. The polymorphonuclear leukocytes were enumerated in a hemocytometer and resuspended in PBS to give a final concentration of 3×10^7 cells/ml. This procedure typically yielded a cell preparation which consisted of 80% polymorphonuclear leukocytes with the major contaminating cell being the non-phagocytic lymphocyte. Viability was greater than 95% as judged by the ability of the cells to exclude trypan blue dye.

Zymosan was opsonized by incubating 50 mg for 30 minutes in 1.0 ml PBS containing 2.0 ml pooled human serum. The zymosan was then pelleted by centrifugation at 27,000 xg for 15 minutes and resuspended in 3.0 ml PBS for use in the assays. Zymosan-activated serum, employed as a control in some experiments, consisted of the supernatant solution obtained from the 27,000 xg centrifugation.

The neutrophils (3×10^7 segmented cells in 1.0 ml PBS) were incubated with [^{3}H] arachidonate and [^{14}C] palmitate in a 25 ml flask for 2 hours. The radiolabeled fatty acids complexed to defatted bovine serum albumin (6) were added to give $2.8\text{-}4.2 \times 10^5$ cpm [^{3}H] and $1.1\text{-}2.1 \times 10^5$ cpm [^{14}C]. The amount of albumin added was 10 to 60 µg. Following the incubation the cells from each flask were transferred to a 12 ml conical centrifuge tube, the flask was washed with 1.0 ml PBS and the wash added to the cells. Following centrifugation of the cells at 250 xg for 8 minutes and a wash with 5 ml PBS, the cells were resuspended in 0.5 ml PBS. To each flask either 0.5 ml PBS (resting) or 0.5 ml PBS that contained opsonized zymosan (phagocytizing) was added. The tubes containing the cells were then incubated at 37°C in a shaker for the times indicated in each experiment. The incubations were terminated by the addition of 3 ml $CHCl_3$-methanol (1:2), the lipids extracted by the method of Bligh and Dyer (7) and the products separated by thin-layer chromatography on Silica gel H. The neutral lipids were separated in the chloroform-methanol-acetic acid-water (75:48:12.5:4.5) system (System 1) while the phospholipids were separated by the hexane-ether-formic acid (90:60:6) system (System 2). The compounds were visualized by I_2 vapor, scraped into a scintillation vial and counted in a toluene-Triton X-100-water solvent (2:1:0.2) containing Omniflor.

Materials. The [5,6,8,9,11,12,14,15-^{3}H] arachidonate (5 Ci/mmol) and the [1-^{14}C] palmitate (56 mCi/mmol) were purchased from Amersham/Searle, Des

Plains, Ill., bovine serum albumin (Fraction V) and zymosan A were from Sigma, St. Louis, Mo., and the Omniflor was obtained from New England Nuclear, Boston, Mass. Plasma gel was obtained from the HTI Corp., Buffalo, N. Y., and phorbol myristate acetate (PMA) from the Consolidated Midland Corp., Brewster, N. Y. The PMA was stored frozen as a stock solution of 2 mg/ml in dimethyl sulfoxide; immediately before use it was diluted to the appropriate concentration with PBS. Control experiments indicated that the small amount of DMSO present in the final incubation had no effect on neutrophil viability or function.

Results. Preliminary experiments showed that more than 95% of the [^{3}H] arachidonate and 90% of the [^{14}C] palmitate were incorporated into the cellular lipids during the 2 hour pre-incubation. Nearly 50% of both labels was incorporated into phosphatidylcholine, the rest being distributed between the triacylglycerol, phosphatidylethanolamine, and sphingomyelin/lysophosphatidylcholine fractions; very little was in the phosphatidylserine/phosphatidylinositol fraction (Table 1). This pattern did vary somewhat between experiments, the main change was higher percentages in phosphatidylethanolamine and phosphatidylserine/phosphatidylinositol with concomitant decreases in triacylglycerol and phosphatidylcholine. The data presented here are from an experiment that is representative of eight done. In all experiments there was a rapid release of [^{3}H] arachidonate upon the challenge with opsonized zymosan (Table 1; 1.3 to 7.4%). This was not found with [^{14}C] palmitate (8.1 to 8.3%). In other experiments we found that resting cells were identical to those extracted prior to incubation. Concomitant with the release of [^{3}H] arachidonate was a decrease in the amount of [^{3}H] phosphatidylcholine (48.5 to 41.2%) in all experiments. The [^{14}C] content of the phosphatidylcholine did not decrease nor was there an increase in the amount of [^{14}C] lysophosphatidylcholine. These results suggest that the precursor for the [^{3}H] arachidonate released is phosphatidylcholine. The other product expected from the action of a phospholipase A_2, lysophosphatidylcholine, was not found; this could be the result of a rapid reacylation of the lysophosphatidylcholine by a non-labeled fatty acid, however. To insure that no [^{14}C] lysophosphatidylcholine remained in the methanol H_2O layer after extraction, we lyophilized that layer and chromatographed the redissolved residue; no [^{14}C] lysophosphatidylcholine was found. Further,

Table 1

Distribution of [^{3}H] Arachidonate and [^{14}C] Palmitate Following 5 Minute Challenge

Compound	Addition			
	None		Opsonized zymosan	
	[^{3}H]	[^{14}C]	[^{3}H]	[^{14}C]
System 1				
Cholesterol ester	1.4	3.1	1.8	4.5
Triacylglycerol	19.0	28.5	19.5	25.7
Fatty acid	1.3	8.1	7.4	8.3
Diacylglycerol	2.0	3.4	1.7	3.4
Monoacylglycerol	0.4	4.7	0.5	4.6
System 2				
Phosphatidylethanolamine	12.4	4.1	12.9	4.0
Phosphatidylserine/phosphatidylinositol	1.9	1.4	1.7	1.6
Phosphatidylcholine	48.5	41.5	41.2	39.1
Sphingomyelin/lysophosphatidylcholine	12.4	11.0	12.8	10.7

The conditions of the experiment are given in Methods. The total amount of radiolabel recovered in each sample was as follows: [^{3}H] = .97 to 1.13 x 10^{5} cpm; [^{14}C] = 5.1 to 5.7 x 10^{4} cpm. (No correction was made for counting efficiency, only spillover. This accounts for difference between the recovery on thin-layer chromatography and original radioactivity.) The results are expressed as the percentage in each compound, relative to the total recovered from the plates. Total phospholipid in System 1 and total neutral lipid in System 2 are not presented.

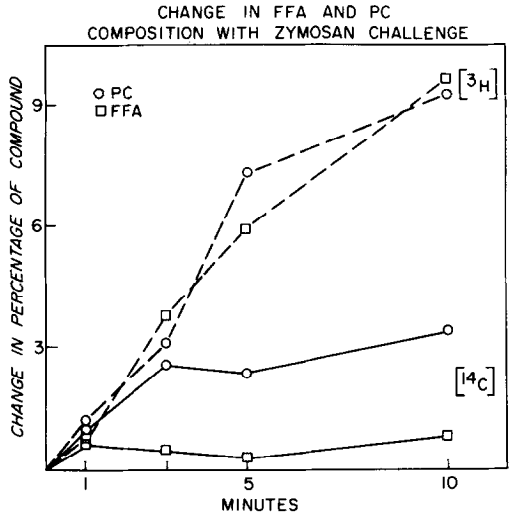

Figure 1. The conditions of the experiment are given in Methods. The changes in free fatty acid (FFA) are increases whereas those for phosphatidylcholine (PC) are decreases. The solid lines are for [^{14}C] and dashed lines are for [^{3}H].

we detected no appreciable oxidation of [^{14}C] palmitate to [^{14}C] O_2. In two experiments we found an increase in monoacylglycerol during phagocytosis (up to 8% of both labels) that suggests that pathways other than a direct deacylation of phosphatidylcholine or other substrates might be involved. Since this is not a reproducible finding more experimentation is necessary to resolve this point.

The kinetics of the release of [^{3}H] arachidonate are shown in Fig. 1, the release increased up to 10 min with a concomitant decrease in [^{3}H] phosphatidylcholine. In this, but not all experiments, there was a slight decrease in [^{14}C] phosphatidylcholine. This amounted to a decrease from 41.5 to 39.1% and probably is not significant since there was no change in the [^{14}C] lysophosphatidylcholine content (Table 1) or [^{14}C] palmitate (Table 1 and Fig. 1). In other experiments not presented, the release of [^{3}H] arachidonate did not proceed significantly beyond 10 min.

To show that the release of [^{3}H] arachidonate was specific for the opsonized zymosan, several controls were carried out and compared with the resting cells (Table 2). These experiments demonstrate that the zymosan must be opsonized (column 2) and that the serum removed from the opsonized zymosan does not have

Table 2

Requirement for Opsonized Zymosan to Release [^{3}H] Arachidonate

Precursor	Percentage of resting					
	Opsonized zymosan	Zymosan control	Zymosan activated serum	Serum	Heat inactivated serum	PMA
[^{3}H] Arachidonate	292	80	92	100	72	100
[^{14}C] Palmitate	122	100	77	78	65	79

The conditions of the experiment are given in Methods. The incubation time was 3 minutes, and the additions are as follows: phorbol myristate acetate (PMA), 1 µg; all serum fractions, 50 µl (an amount in large excess of that which could be added with opsonized zymosan); control zymosan, equal to opsonized zymosan. The zymosan activated serum was the supernatant from the zymosan after centrifugation. The results are presented as the percentage of the resting value (resting = 100%).

lipolytic activity (column 3). Further, neither fresh nor heat-inactivated serum (columns 4 and 5, respectively) caused a change in the amount of [^{3}H] arachidonate recovered. In order to determine if the release of [^{3}H] arachidonate is related to initiation of the respiratory burst we used 1.0 μg/ml phorbol myristate acetate (PMA), a compound known to initiate the burst without stimulating phagocytosis (8). As can be seen in column 6, PMA was without effect. In all cases no significant change was noted in the amount of [^{14}C] palmitate recovered.

Discussion. The results presented here demonstrate that [^{3}H] arachidonate is released from phosphatidylcholine in response to challenge by opsonized zymosan. It is likely that this is the result of the stimulation of a phospholipase A_2. Indeed such an enzyme in neutrophils was postulated by Hirata and coworkers to be stimulated by the chemoattractant tripeptide f-Met-Leu-Phe (9). In those experiments, as in ours, no evidence was presented to indicate that lysophosphatidylcholine, the other product of phospholipase A_2 action, was produced. Indeed, f-Met-Leu-Phe caused a decrease in the total amount of methylated lipids. It is possible that other lipolytic sequences are involved such as the combined action of a phospholipase A_1 and a lysophospholipase. However, the data presented here do not support such a possibility.

In a number of studies on other types of cells it has been shown that the release of arachidonate from membranous lipid is the first and rate limiting step in prostaglandin synthesis (10). Indeed, recent reports by Borgeat and Samuelsson (11,12) demonstrate that arachidonate, when added to neutrophils, was converted to hydroxylated derivatives. These authors suggested that these acids are formed by lipoxgenase-type reactions, similar to that in platelets (13). Indeed Goldstein et al. (14) demonstrated the generation of thromboxane A_2 by neutrophils exposed to opsonized zymosan. These studies, however, employed exogenously added arachidonate and thus did not address the question of the physiologic origin of the arachidonate. The ionophore A 23187 stimulated

the release of endogenous presursor (presumably arachidonate) through the purported release of lysosomal enzymes. Although not rigorously studied, under the conditions employed no evidence was found for the production of hydroxylated derivatives of arachidonate, based on the thin-layer chromatographic analysis.

The question arises as to whether the release of arachidonate is related to phagocytosis per se, or to the subsequent metabolic events which accompany phagocytosis. The evidence presented here is suggestive, but not conclusive. The addition of 1 μg/ml of PMA to resting neutrophils has been demonstrated to cause a complete burst in oxidative metabolism similar to that which accompanies phagocytosis (8) as well as degranulation of the specific granules to the exterior of the cell (15). We were unable to document the release of arachidonate from pre-labeled cells in the presence of amounts of PMA which maximally stimulate the cell metabolically. This suggests, but does not prove, that the release of free fatty acid is related to the phagocytic event rather than to the subsequent metabolic alterations of the cell. However, Kakinuma has demonstrated that exogenous free fatty acids under some conditions are capable of altering neutrophil oxidative metabolism (16). Clearly, further work is required to firmly establish the role of endogenous arachidonic acid release during phagocytosis.

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