Lipids of Alveolar Macrophages, Polymorphonuclear Leukocytes, and Their Phagocytic Vesicles

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ABSTRACT Phagocytic vesicles were isolated from rabbit alveolar macrophages and guinea pig polymorphonuclear leukocytes that had ingested emulsified paraffin oil. Phospholipids and their fatty acids were determined in whole cells and in the phagocytic vesicle and pellet fractions separated from them. The cholesterol-to-phospholipid ratios in the vesicle fractions were distinctly higher than those of the respective whole cells or pellet fractions. The vesicle fractions also had higher phospholipid-to-protein ratios than did the whole cells. The phospholipids of the phagocytic vesicle fraction from macrophages contained relatively more sphingomyelin, lyso-(bis)phosphatidic acid, and phosphatidylserine and less lecithin, phosphatidylethanolamine, and phosphatidylinositol than did the whole cells or pellet fractions. The phospholipids of phagocytic vesicles from polymorphonuclear leukocytes contained significantly more phosphatidylinositol than did the pellet fractions. Lyso(bis)phosphatidic acid, which constituted 15% of the phospholipid in rabbit alveolar macrophages and 25% of that in their phagocytic vesicles, contained almost 60% oleic acid and 20% linoleic acid. This lipid was not detected in rabbit peritoneal macrophages or in rat alveolar macrophages.

The polyunsaturated fatty acids of leukocyte phospholipids were chiefly linoleic, whereas in macrophages arachidonic accounted for almost 20% of the total fatty acids. The macrophages produced malondialdehyde when ingesting polystyrene beads or emulsified paraffin oil, from which it was inferred that peroxidation of endogenous lipid can occur during phagocytosis. Polymorphonuclear leukocytes in which less than 3% of phospholipid fatty acids were arachidonic did not produce malondialdehyde during phagocytosis of these inert particles, but did when ingesting an emulsion containing linolenate, thus providing evidence for peroxidation of ingested lipid. Isolated phagocytic vesicles from alveolar macrophages contained lipid peroxides and generated malondialdehyde when incubated with ADP, $FeCl_3$, and NADH.

INTRODUCTION

During formation of phagocytic vesicles in mammalian cells, fusion of cytoplasmic granules with the vesicle membrane results in discharge of granule enzymes, which includes an acid lipase (1, 2) and phospholipase (3). These enzymes, and also the hydrogen peroxide generated during phagocytosis (4, 5), may have effects on the lipids of the vesicle membrane as well as on those of the ingested microorganisms. Alterations of lipid metabolism associated with phagocytosis include increased rates of incorporation of acetate into mixed cellular lipids (6) and of phosphate into certain acidic phospholipids (7, 8) by polymorphonuclear leukocytes (PMN).[1] Elsbach observed that acylation of exogenous lysolecithin was stimulated (9) and there was transfer of fatty acids from triglycerides to phospholipids during phagocytosis (10). With the exception, however, of reports by Elsbach (11) concerning rabbit PMN and Gottfried (12) on human leukocytes, there has been a dearth of information concerning the lipids of mammalian phagocytic cells. We present below the results of an investigation of phospholipids and their fatty acids in guinea pig PMN, rabbit alveolar macrophages, and phagocytic vesicles isolated from these cells. An unusual

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[1] *Abbreviations used in this paper:* BHT, 2,6-di-*tert*-butyl-*p*-cresol; GLC, gas-liquid chromatography; PMN, polymorphonuclear leukocytes; TLC, thin-layer chromatography.

lipid, which accounted for 15% or more of the phospholipid phosphorus in rabbit alveolar macrophages and constituted 25% of the phospholipid of the phagocytic vesicles, was isolated and identified as lyso(bis)phosphatidic acid. In addition, we have observed peroxidation of endogenous lipid by alveolar macrophages during phagocytosis and by phagocytic vesicles from these cells incubated with ADP, $FeCl_3$, and NADH. Evidence for peroxidation of lipid ingested by PMN was also obtained.

METHODS

Preparation and fractionation of cells. Alveolar macrophages were obtained by pulmonary lavage with 150 mM NaCl from 3- to 4-kg male rabbits that had been injected intravenously 3.5-4.5 wk earlier with 1 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) (stimulated macrophages) (13). Guinea pig PMN were collected from peritoneal exudates as previously described (14). Alveolar macrophage and leukocyte preparations were more than 90% pure by differential counts of Giemsa stained smears. Cells were suspended in Krebs-Ringer phosphate medium, modified to contain 0.92 mM $CaCl_2$ at a final pH of 7.4. The medium contained 5.5 mM glucose and bovine serum albumin, 1 mg/ml. Before isolation of phagocytic vesicles, cells were incubated for 45 min at 37°C with an albumin-paraffin oil emulsion (15), 0.2 ml/ml total volume. Control cells were incubated with a corresponding amount of the albumin solution used to make the emulsion. After incubation, the control cells (designated "cells" in the tables) were sedimented by centrifugation and extracted with chloroform-methanol, 2:1 (v/v), containing 50 μg/ml BHT (2,6-di-*tert*-butyl-*p*-cresol) (a gift from Shell Oil Company, New York). Cells incubated with the emulsion were washed with Dulbecco's phosphate-buffered saline (containing per liter 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4) and then disrupted with a tight-fitting Dounce homogenizer in a solution containing 0.36 M sucrose, 1 mM Tris, pH 7.5, and sodium heparin, 500 U/ml. Homogenization was continued until at least 90% of the cells were broken as determined by phase microscopy. Subcellular fractions were prepared from alveolar macrophages (16) and PMN (15) as previously described, except that 0.15 M NaCl was used for the upper gradient layer in some experiments because it improved the packing of the macrophage vesicle fraction. The pellet and floating phagocytic vesicle fractions were dispersed by homogenization in 0.15 M NaCl. Samples were removed for protein determination (17) and for extraction with chloroform-methanol containing BHT as described above. The supernatant fluid fractions, which contained about 6% of the phospholipid phosphorus of the alveolar macrophages and less than 1% of that of PMN, were discarded.

Lipid analysis. Lipids were extracted overnight. The extracts were filtered, washed with 100 mM KCl (18), and taken to dryness under nitrogen. Extracted lipids were stored in chloroform-methanol, 2:1 (v/v), at −20°C until analyzed. After saponification, cholesterol was purified by thin-layer chromatography (TLC) and quantified by gas-liquid chromatography (GLC) (15). The paraffin oil did not interfere with the lipid analyses and was easily separated from cell lipids by running the thin-layer plates in hexane-diethyl ether, 95:5 (v/v), before chromatography for separation of neutral lipids (19). Phospholipids were separated by two-dimensional chromatography (20) on 250 μ silica H plates (Analtech, Inc., Wilmington, Del.) after initial development with hexane-diethyl ether, 60:40, to remove paraffin oil. The plates were sprayed with a mixture of anisaldehyde, glacial acetic acid, and sulfuric acid, and the spots identified by comparison with known standards. Phosphorus was measured by the method of Bartlett (21).

Lecithin and phosphatidylethanolamine fractions were isolated by TLC and eluted as previously described (22). Phospholipid bands were identified after a brief exposure of plates to iodine vapor. (The fatty acid composition of rat liver phosphatidylethanolamine briefly exposed to iodine vapor in this manner was not different from that found when 2,7-dichlorofluorescein was used for visualization.) Because the phosphatidylethanolamine fraction was occasionally contaminated with lyso(bis)phosphatidic acid and some neutral lipids, it was purified further by chromatography using chloroform-methanol-water-15 M NH_4OH, 80:36:1.7:0.3 (v/v). Lecithins were divided into disaturated and unsaturated species by chromatography after formation of mercuric acetate adducts (22).

For analysis of fatty acid composition, total phospholipids were isolated by silicic acid chromatography (23). The phospholipid fraction was transesterified (in the presence and absence of BHT) with 14% BF_3 in methanol (Applied Science Laboratories, Inc., State College, Pa.) for 90 min at 100°C to ensure complete transesterification of sphingomyelin (24). Methyl esters of the fatty acids of phosphatidylethanolamine and lecithin were prepared after mild alkaline hydrolysis by esterification in 1.5% (v/v) sulfuric acid in methanol in the presence of BHT (25). The methyl esters were separated from the BHT and dimethylacetals by TLC in benzene on 250 μ Silica G plates (Analtech, Inc., Wilmington, Del.), prestained with Rhodamine-6-G (Applied Science Laboratories) and eluted with chloroform (24). (Methyl esters prepared by transesterification with BF_3 in methanol in the presence of BHT were contaminated by substances, most of which chromatographed with 17:0 methyl ester and were not removed by TLC, but because of their retention times did not interfere with the subsequent analysis.) Fatty acid methyl esters were quantified by GLC isothermally at 176°C and at 184°C on a diethylene glycol succinate column (15% on 80/100 Gas-Chrom P, Laboratories, Inc., State College, Pa.). Retention times were determined with commercial standards for all fatty acids listed. Analyses of National Heart Institute fatty acid standards (KD, KF) agreed with the stated composition with a relative error of less than 5% for the major components (more than 10% of the total mixture) and of less than 15% for minor components (less than 10% of the total mixture). The only methyl esters that were not readily separated were those of 18:3 and 20:1, 20:4 and 22:1. These pairs of fatty acids were separated by chromatographing representative samples on a nonpolar column (mixture of 1 and 3% SE-30 on 80/100 Gas-Chrom Q, Applied Science Laboratories). It was established that the fatty acid designated 20:4 in the tables was in fact arachidonic, that 18:3 rather than 20:1 was present in the diet, and that at least $\frac{2}{3}$ of the methyl ester from cellular lipids designated as 20:1 was in fact 20:1 and not 18:3.

Production of malondialdehyde by cells and isolated phagocytic vesicles. Alveolar macrophages or PMN were incubated with or without polystyrene beads, 11 mg/ml, or paraffin oil emulsion, 0.2 ml/ml total incubation, in siliconized flasks at 37°C. At the indicated times, samples were

removed and added to 0.5 vol of ice-cold 35% (w/v) trichloroacetic acid solution. Malondialdehyde in the acid extracts was quantified with thiobarbituric acid (26). The pink reaction product had the spectrum reported to be characteristic for that of malondialdehyde. Turbidity due to polystyrene was removed by passage of the final soluton through a Millipore filter (0.5 μ) (Millipore Corp., Bedford, Mass.). When paraffin oil was present, chloroform, 2 ml, was added before the final centrifugation to clarify the extracts before spectrophotometry. Addition of catalase to the incubation medium (in case hydrogen peroxide might produce a similar product with thiobarbituric acid) did not alter the results.

Phagocytic vesicles were prepared as previously described (15), but the upper wash layer through which the oil-laden vesicles floated during centrifugation was 0.15 M NaCl, 10 mM Tris (pH 7.4), instead of 0.25 M sucrose, 1 mM Tris. The vesicle fractions were dispersed in 0.15 M NaCl, 10 mM Tris buffer, pH 7.4, and frozen. They were either thawed and used immediately or stored for several days at −70°C. Included in a total incubation volume of 0.5 ml was 0.8–2.4 mg vesicle protein, 4 mM ADP, 0.012 mM $FeCl_3$, and in some instances 0.3 mM NADH or NADPH (27). After incubation at 30°C for the designated period of time, 0.25 ml of cold 35% trichloroacetic acid was added, and malondialdehyde quantified as described above.

Lipid peroxides. Cells and cell fractions were extracted for 30 min at 20°C with chloroform-methanol, 2:1, without BHT, and analyzed immediately. Lipid peroxides were identified by TLC (28) and in other samples were quantified iodometrically under nitrogen (29).

Materials. All solvents were reagent grade (J. T. Baker Chemical Co., Phillipsburg, N. J.). Hexane and chloroform used in preparation of the methyl esters for GLC were redistilled in an all-glass apparatus. Lipid standards used for the characterization of lyso(bis)phosphatidic acid were purchased from Supelco, Inc., Bellefonte, Pa. Cholesterol linolenate and trilinolenin were purchased from Applied Science Laboratories, Inc., State College, Pa. Polystyrene beads (1.1 μ) purchased from Dow Chemical Co., Midland, Mich., were dialyzed extensively against distilled water before use. ADP was purchased from Sigma Chemical Co., St. Louis, Mo., NADH and NADPH from Calbiochem, Los Angeles, Calif., and 2-thiobarbituric acid from Eastman Organic Chemicals, Rochester, N. Y.

RESULTS

Lipid composition of phagocytic vesicles. In previous studies we found that the phagocytic vesicle fractions from alveolar macrophages contained about 9% of the recovered phospholipid phosphorus (16), and those from PMN 18% (15). These fractions have been shown to consist of membrane-bounded vesicles containing electron-dense material that resembles granule matrix. They contain granule-associated enzymes and are not significantly contaminated by nuclear, microsomal, or mitochondrial structures (15, 16). As shown in Table I, the phagocytic vesicle fractions from both types of cells had cholesterol-to-phospholipid ratios that were higher than those of the whole cells or pellet fractions. Phospholipid-to-protein ratios were distinctly higher in the phagocytic vesicles than they were in the respective whole cells, but not higher than those of the pellet fractions. It should be noted, however, that the protein in the vesicle fraction included albumin from the emulsion. Thus the true ratios in the vesicles were somewhat higher than those in Table I. In previous studies (15) albumin constituted about 25% of the protein of the PMN vesicle fraction. The amount of albumin in phagocytic vesicles from alveolar macrophages has not been determined.

Most of the cell triglyceride was found in the floating phagocytic vesicle fraction (data not shown). The free fatty acid content of fractions subjected to homogeniza-

TABLE I

Phospholipid-Protein and Cholesterol-Phospholipid Ratios of Cells and Subcellular Fractions

Fraction	Alveolar macrophage		Polymorphonuclear leukocyte	
	Phospholipid* Protein	Cholesterol‡ Phospholipid	Phospholipid* Protein	Cholesterol§ Phospholipid
Cell	7.35±0.50	0.26±0.01	4.15±0.10	0.54±0.01
Pellet	14.89±0.47	0.22±0.02	10.80±0.60	0.51±0.01
Phagocytic vesicle	12.58±0.56	0.36±0.03	9.48±0.60	0.69±0.01

* Mean ±SE of micrograms lipid phosphorus per milligram protein for each fraction based on analyses of alveolar macrophages from five rabbits and three preparations of guinea pig PMN. The protein of the phagocytic vesicle fractions included albumin from the paraffin oil emulsion. In earlier studies it was calculated that albumin contributed about 25% of the protein in the PMN vesicle fraction (15). The phospholipid content of the albumin used was negligible.

‡ Mean ±SE of moles cholesterol per mole lipid phosphorus for each fraction based on analyses of cells from four rabbits.

§ Mean ± one-half the range of analyses of two prepreparations of guinea pig PMN.

TABLE II

Phospholipid Composition of Cells and Subcellular Fractions

Phospholipid	Alveolar macrophage			Polymorphonuclear leukocyte		
	Cell	Pellet	Phagocytic vesicle	Cell	Pellet	Phagocytic vesicle
Origin	0.7±0.2	0.7±0.2	1.3±0.4	0.5±0.1	0.3±0.1	0.8±0.2
Lysolecithin	0.7±0.2	0.3±0.1	1.5±0.3	0.6±0.1	0.7±0.2	0.9±0.2
Sphingomyelin	13.5±0.7	13.8±0.5	21.9±0.8	18.7±0.4	19.3±0.7	21.2±0.8
Lecithin	32.8±1.0	33.6±0.9	17.8±0.5	33.5±0.4	30.2±0.7	29.5±1.0
Phosphatidyl serine	5.8±0.3	5.7±0.3	7.6±0.5	8.2±0.2	10.7±0.6	9.4±0.5
Phosphatidyl inositol	5.9±0.4	5.8±0.4	3.6±0.4	5.1±0.2	4.4±0.3	5.9±0.4
Phosphatidyl ethanolamine	22.7±0.6	21.9±0.5	19.4±0.3	32.0±0.6	33.6±0.8	31.6±0.4
Polyglycerol phosphate	0.9±0.4	1.0±0.3	0.1±0.1	1.2±0.2	0.9±0.3	0.8±0.5
Lyso(bis)phosphatidic acid	16.9±2.0	17.1±2.1	26.7±2.6	—	—	—

The values (mean ±SE) are the per cent of the total lipid phosphorus recovered. All areas reactive to the anisaldehyde-acetic acid-sulfuric acid spray were assayed. Recovery of phospholipid from alveolar macrophages applied to the plates was 94.2±1.5% (mean ±SE, n=4). Five different preparations from each type of cell were analyzed at least in duplicate. The polyglycerol phosphate had an R_f similar to that of diphosphatidylglycerol and was not further characterized.

tion and density-gradient centrifugation was greater than that of cells (control or after ingestion or paraffin oil) extracted directly. As shown in Table II, phospholipids of the phagocytic vesicle fraction from alveolar macrophages contained relatively more sphingomyelin, lyso(bis)phosphatidic acid, and phosphatidylserine, and less lecithin, phosphatidylethanolamine, and phosphatidylinositol than did the whole cells or pellet fractions. No attempt was made to quantify the plasmalogen component of the phospholipids. The phospholipid composition of the phagocytic vesicles from PMN was only slightly different from that of the whole cells or pellet fractions, although they were significantly enriched in phosphatidylinositol ($P < 0.05$ for mean of differences between vesicle and pellet fractions of each homogenate).

There were marked differences between the PMN and alveolar macrophages in terms of fatty acid composition of total and individual phospholipids (Tables III and IV). The macrophage phospholipids had a much higher percentage of 20:4 than did those of the PMN, and the phosphatidylethanolamine of both pellet and vesicle fractions contained significant amounts of 22:5 and 22:6 which were not present in more than trace amounts in any PMN lipids studied. The fatty acids of the phospholipids of the phagocytic vesicles from both

TABLE III

Fatty Acid Composition of Alveolar Macrophage Phospholipids

Fraction	14:0*	16:0	16:1	18:0	18:1	18:2	20:1	20:4	24:1	22:5	22:6
	percentage of total fatty acids										
Total phospholipid											
Cell (2)	0.6±0.1	15.7±2.4	1.7±0.2	16.5±1.2	31.5±3.9	13.4±1.2	0.6±0.1	18.7±0.8	1.1±0.9	‡	‡
Pellet (2)	0.7±0.2	16.4±2.1	1.7±0.1	16.3±0.1	30.8±0.9	13.1±1.2	0.6±0.1	18.6±0.2	1.6±0.3	‡	‡
Vesicle (2)	1.5±0.3	21.7±1.8	1.7±0.2	20.4±2.0	28.7±3.4	9.7±1.5	0.6±0.1	14.6±0.6	1.6±0.4	‡	‡
Phosphatidylethanolamine											
Pellet (4)	0.5±0.1	4.5±0.2	1.4±0.1	21.1±0.9	21.5±0.9	7.3±0.7	0.4±0.1	32.0±1.0	‡	2.7±0.4	8.5±1.4
Vesicle (4)	1.2±0.3	11.3±1.1	1.8±0.2	23.0±1.3	26.4±1.3	6.7±1.1	0.7±0.1	22.7±2.3	‡	1.6±0.3	3.8±0.7
Lecithin											
Pellet (4)	1.1±0.1	28.9±0.6	6.6±0.4	10.2±0.2	21.5±0.7	16.7±1.1	0.3±0.1	14.8±0.5	‡	‡	‡
Vesicle (4)	0.4±0.1	34.0±1.5	5.6±0.5	14.0±1.1	22.0±1.7	12.4±0.5	0.4±0.2	11.2±0.7	‡	‡	‡
Lyso(bis)phosphatidic acid											
Cell (2)	‡	3.2±0.5	1.4±0.3	7.1±0.2	58.1±3.8	22.1±2.6	1.0±0.3	7.0±1.1	‡	‡	‡

Values presented are mean ±SE for number of fractions analyzed indicated in parentheses, or mean ± one-half the range when n = 2.
* Chain length:number of double bonds.
‡ Less than 0.5% in all samples.

TABLE IV

Fatty Acid Composition of Polymorphonuclear Leukocyte Phospholipids

Fraction	14:0	16:0	16:1	18:0	18:1	18:2	20:1	20:2	20:4	24:1
					percentage of total fatty acids					
Total phospholipid										
Cell (4)	0.6±0.2	17.5±1.2	0.6±0.2	23.2±1.0	21.6±0.7	27.6±0.4	2.2±0.4	1.7±0.8	2.7±0.7	2.0±0.9
Pellet (4)	0.6±0.1	17.9±0.7	0.6±0.1	21.4±0.5	21.8±0.4	29.2±0.4	2.2±0.2	1.0±0.3	2.4±0.2	3.5±0.5
Vesicle (4)	0.6±0.2	22.3±0.9	0.9±0.1	25.4±1.9	22.9±1.3	23.7±1.1	1.9±0.5	0.8±0.1	0.7±0.4	2.1±0.7
Phosphatidylethanolamine										
Pellet (4)	0.3±0.1	6.1±0.1	0.7±0.1	27.2±1.1	27.8±0.4	31.6±0.9	2.4±0.3	0.8±0.4	3.7±0.2	*
Vesicle (4)	0.3±0.1	9.1±1.7	0.6±0.5	29.0±1.5	28.2±1.0	27.2±2.1	2.3±0.3	1.0±0.3	2.6±0.1	*
Lecithin										
Pellet (3)	0.7±0.3	24.0±0.5	1.4±0.2	14.7±0.5	19.9±0.6	33.5±0.7	3.9±0.1	1.0±0.3	0.9±0.4	*
Vesicle (3)	1.4±0.3	29.2±1.1	1.7±0.2	14.3±0.7	20.0±0.1	29.6±0.8	3.1±0.2	0.6±0.1	0.4±0.1	*

Data are presented as in Table III.
* Less than 0.5% in all samples.

types of cells tended to be more saturated and less polyunsaturated than those from the whole cells or from the pellet fractions. In addition, in both types of cells, the percentage of the lecithins that were disaturated was significantly greater in phagocytic vesicles than in whole cells or the pellet fractions (Table V). In both types of cells, the fatty acids of the disaturated lecithins were mostly palmitic (77% in alveolar macrophages and 65% in PMN). Phagocytosis of polystyrene beads caused no increase in the rate of incorporation of radioactive glycerol or palmitate into disaturated lecithins or in the distribution of labeled palmitate between the 1- and 2-positions (data not shown). Incorporation of lysolecithin-^{32}P into total lecithins was doubled in cells ingesting paraffin oil emulsion, but the incorporation into disaturated lecithins as percentage of the total was unchanged.[2]

Lyso(bis)phosphatidic acid. An unusual phospholipid accounted for about 15% of the phospholipid phosphorus in both stimulated and normal rabbit alveolar macrophages, but was not found in PMN. The migration of this lipid was similar to that of phosphatidylglycerol in most TLC solvent systems, but the two lipids migrated differently in cholorform-methanol-water-15 M NH_4OH, 80:36:1.7:0.3, and in diisobutyl ketone-acetic acid-water, 80:50:7.4. The initially unidentified lipid was ninhydrin negative whether isolated with acidic or basic chromatography solvents from cells extracted at neutral or acidic pH. When this lipid and authentic phosphatidylglycerol were deacylated by mild alkaline hydrolysis, passed through a column of Dowex-50 H^+ (Dow Chemical Co., Midland, Mich.), and silylated, a single glycerol base identified as glycerophosphoryl glycerol by GLC mass spectrometry (30) was obtained from each of these compounds. The unknown lipid, phosphatidylglycerol, cardiolipin, lecithin, lysolecithin, 1-monoolein, 2-monoolein, and glycerol were converted to their glycerol acetates by acetolysis with acetic anhydride-acetic acid, 2:3, for 4 hr at 140°C (31). Acetolysis of the unknown yielded only one compound which was identified as monoacyl glycerol acetate by TLC and GLC mass spectroscopy. This monoacyl glycerol acetate isolated by TLC with hexane-diethyl ether-acetic acid, 65:35:1, was shown to be predominantly 2-acyl glycerol acetate by its nuclear magnetic resonance spectrum in benzene which was compared to spectra of the derivatives of 1- and 2-monoolein. Acetolysis under conditions vigorous enough to convert lyso(bis)phosphatidic acid to glycerol acetate caused considerable isomerization of monoglycerides. The 1- and 2-monoolein standards were, therefore, prepared by reacting the respective monoglycerides with acetic anhydride in pyridine.

TABLE V

Disaturated Lecithins in Cells and Subcellular Fractions

	Alveolar macrophage	Polymorphonuclear leukocyte
	Percentage of total lecithins	
Cells	13.9±0.9	15.6±0.2
Pellet	12.5±0.5	13.4±0.2
Vesicle	22.2±1.3	18.7±1.3

Values presented are means ±SE for analyses of alveolar macrophages from five rabbits and PMN from four guinea pigs. The phagocytic vesicle fractions from both types of cells had a significantly higher percentage of disaturated lecithins than did whole cells or pellet fractions ($P < 0.01$ for mean of differences between vesicles and cells or pellet for each preparation).

[2] Unpublished experiments carried out in collaboration with Doctors Peter Elsbach and Pierluigi Patriarca.

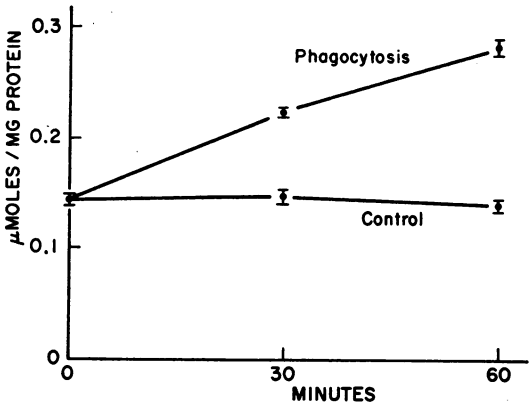

FIGURE 1 Production of malondialdehyde by alveolar macrophages incubated with polystyrene beads. Rabbit alveolar macrophages (6.5 mg cell protein/ml) were incubated with or without polystyrene beads, and at the indicated times, samples (cells plus medium) were removed for determination of malondialdehyde, which is expressed as micromoles/milligram cell protein. The mean and range of duplicate analyses are presented.

All of the evidence is compatible with the designation of this lipid as lyso(bis)phosphatidic acid, although it appears that most of the fatty acids are on the 2 and 2′ positions. The ester (32) to phosphorus ratio was 2.0:1.1 (expected 2:1). The composition of the fatty acids liberated by alkaline hydrolysis is shown in Table III. No unusual fatty acids were found by GLC on polar and nonpolar columns. Lyso(bis)phosphatidic acid, which has previously been found only as a minor component in normal mammalian tissues, accumulates in large amounts in patients with Niemann-Pick disease (33, 34). The lipid isolated from alveolar macrophages co-chromatographed with lyso(bis)phosphatidic acid from liver and lung of such a patient.

Lipid peroxidation. Alveolar macrophages contained malondialdehyde and generated more during phagocytosis of polystyrene beads (Fig. 1) or paraffin oil emulsions (data not shown). After incubation with polystyrene beads, 70% of the accumulated malondialdehyde was found in the medium. Malondialdehyde was not detected in PMN at rest or after phagocytosis of either polystyrene beads or paraffin oil emulsions. When, however, polymorphonuclear leukocytes were incubated with an emulsion that contained linolenic acid (18:3), malondialdehyde was produced during phagocytosis (Fig. 2).

Phagocytic vesicles from alveolar macrophages that had ingested paraffin oil contained malondialdehyde and generated more when incubated at 30°C with ADP, $FeCl_3$, and NADH, but not NADPH (Fig. 3). Vesicles previously heated at 100°C for 3 min produced no malondialdehyde under these conditions. When the pellet fraction was incubated with ADP and $FeCl_3$, addition of either NADH or NADPH enhanced malondialdehyde formation.

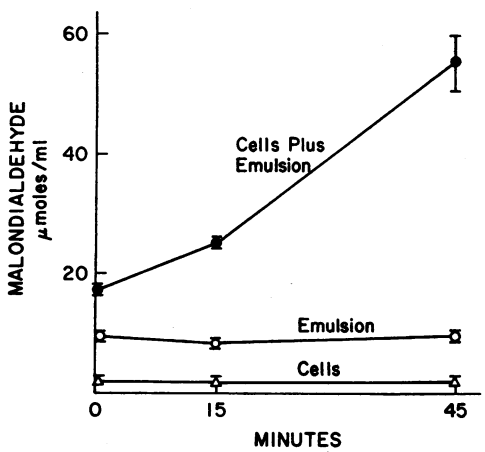

FIGURE 2 Production of malondialdehyde by PMN incubated with emulsion containing cholesterol linolenate and trilinolenin. Cells (12.6 mg protein/ml), emulsion, or both were incubated and samples removed at the indicated times for determination of malondialdehyde. For preparation of the emulsion, cholesterol linolenate and trilinolenin (after removal of the benzene in which they were dissolved with a stream of N_2) were sonified with paraffin oil and buffer containing albumin as previously described (15). During incubation, the concentration of cholesterol linolenate was 75 μg/ml and of trilinolenin 500 μg/ml. Data are presented as in Fig. 1.

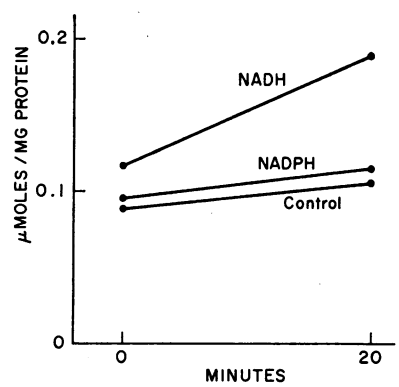

FIGURE 3 Production of malondialdehyde by phagocytic vesicles from alveolar macrophages. Phagocytic vesicles from alveolar macrophages were prepared and incubated as described in Methods. The malondialdehyde content of vesicles plus medium is recorded as micromoles/milligram vesicle protein. Each point represents the mean of values from two or three replicate incubations that agreed closely. Very similar data were obtained in experiments with phagocytic vesicles from two other preparations of macrophages.

Phagocytic vesicles from alveolar macrophages contained a higher concentration of lipid peroxides than did the corresponding cell pellet fractions or the pellet fractions from control cells (120 compared to 15 and 12 μEq peroxide/μg lipid P). Lipid peroxides, which chromatographed as phospholipids, were found in the extracts of the phagocytic vesicle fractions from both alveolar macrophages and PMN and were not detected in the pellet fractions from phagocytizing or control cells carried through the same procedures. Lipid peroxides, which behaved chromatographically as neutral lipids, were found in phagocytic vesicles and in pellet fractions from resting and phagocytizing cells.

DISCUSSION

The cholesterol-to-phospholipid ratios in phagocytic vesicle fractions from both PMN and alveolar macrophages were distinctly higher than those of the respective whole cells or pellet fractions, which is consistent with the fact that the vesicle fractions are relatively enriched in plasma membranes. The phospholipid composition of the vesicles from PMN differed little from that of the whole cells or pellet fractions as might be expected, since most of the phospholipid in these cells is in the plasma and granule membranes. In alveolar macrophages, on the other hand, which contain a variety of cell organelles and residues of previously ingested material, the vesicle fraction contained significantly larger percentages of lysolecithin, sphingomyelin, phosphatidylserine, and lyso(bis)phosphatidic acid than did the whole cells or pellet fractions. The percentages of phosphatidylinositol, phosphatidylethanolamine, and lecithin were lower, but a larger fraction of the lecithins was disaturated. Because they are not susceptible to peroxidation, the disaturated lecithins could be important in stabilization of the phagocytic vesicle structure, but no evidence for a specific or unique effect of phagocytosis on metabolism of disaturated lecithins was obtained.

In both alveolar macrophages and PMN, the phospholipids of the vesicle fractions contained relatively more saturated and less polyunsaturated fatty acids. The polyunsaturated fatty acids of PMN were chiefly 18:2 with only small amounts of 20:2 and 20:4, whereas in alveolar macrophages 20:4 accounted for almost 20% of the phospholipid fatty acids. (Both rabbits and guinea pigs were fed the same diet, the fatty acid composition of which was 19% 16:0, 1% 16:1, 3% 18:0, 50% 18:1, and 27% 18:3 [linolenic].)

The source of the lyso(bis)phosphatidic acid, which constituted about 15% of the phospholipid of rabbit alveolar macrophages and 25% of that in their phagocytic vesicles, remains unclear. It was present in essentially the same concentration in macrophages from rabbits that had not received Freund's adjuvant and in rabbits in Boston and San Francisco as well as in Bethesda, but was not found (less than 2% of lipid phosphorus) in rabbit peritoneal macrophages or in alveolar macrophages from normal or germ-free rats. Rabbit alveolar macrophages incubated with glycerol-U-^{14}C or inorganic phosphate (^{32}P) incorporated no detectable radioactivity into lyso(bis)phosphatidic acid during a period when there was significant incorporation of these precursors into other phospholipids. This may mean only that the rate of synthesis is very low or that the precursor pools are relatively large. On the other hand, this lipid may be a microbial product accumulated in secondary lysosomes that fuse with the newly formed phagocytic vesicles. Lyso(bis)phosphatidic acid has not been described in bacteria which do, however, contain derivatives of phosphatidylglycerol that could perhaps be converted to lyso(bis)phosphatidic acid by macrophages.

Malondialdehyde, a labile aldehyde, is the product of peroxidation of certain unsaturated fatty acids. Because it is easily quantified, malondialdehyde accumulation is used as an index of lipid peroxidation recognizing that peroxidation of all fatty acids will not be detected by this procedure. Alveolar macrophages which contained large amounts of arachidonic acid produced malondialdehyde when ingesting polystyrene beads or emulsified paraffin oil, from which it may be inferred that peroxidation of endogenous lipid can accompany or follow phagocytosis. PMN which contained much less arachidonate did not produce malondialdehyde during or after phagocytosis of the inert particles, but did so when the ingested emulsion contained linolenate, thereby providing direct evidence for peroxidation of ingested lipid.

By iodiometry and TLC, it was demonstrated that phagocytic vesicles from both types of cells contained lipid peroxides and that some of these were apparently phospholipids. The process by which lipid peroxidation occurs is unknown. Both alveolar macrophages and PMN produce hydrogen peroxide (4, 5), and a pyridine-nucleotide oxidase system that catalyzes the peroxidation of endogenous membrane lipids has been demonstrated in rat liver microsomes (27). Lipid peroxidation by isolated phagocytic vesicles was enhanced by NADH but not by NADPH, whereas the pellet fraction of alveolar macrophages (which contains both mitochondria and microsomes) generated lipid peroxides in the presence of either NADH or NADPH. The liver microsomal peroxidation system requires NADPH. In addition to altering cellular and ingested lipids and possibly releasing lysosomal enzymes (35), lipid peroxidation generates aldehydes which may play an important role in bacterial killing (36).

Whether certain characteristics of the lipids of the phagocytic vesicles are due entirely to the concentration of specific membranous structures in this fraction, or are at least in part secondary to alterations in phospholipids or destruction of fatty acids during phagocytosis, cannot be decided. As discussed above, lipid peroxidation is apparently accelerated during phagocytosis, and this process, by destroying polyunsaturated fatty acids, could cause an apparent enrichment in saturated ones. In addition, the possibility of alteration (enzymatic or nonenzymatic) of membrane composition by or during the isolation of vesicles must be considered. In fact, free fatty acids were identified in cell fractions but were not detected in cells extracted directly, i.e., without homogenization and fractionation. The phagocytic vesicles presumably contained phospholipase (3) and acid lipase (1, 2) acquired through degranulation, and effects of these enzymes on vesicle lipids not only cannot be ruled out, but are likely one of the usual concomitants of phagocytosis. In the pellet fractions which contained by far the largest fraction of the lipid phosphorus, the composition of the phospholipids and their fatty acids was very similar to that of the whole cells, from which we infer that no gross changes occurred during phagocytosis and isolation of cell fractions. It seems most probable that enrichment of the vesicle fraction in certain phospholipids is at least in large part due to selection of membranous components that are rich in these compounds.

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