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Phagosomal Membrane Lipids of LM Fibroblasts

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Summary. Murine fibroblasts, LM cells, were cultured in suspension or monolayer in a chemically defined medium without serum and exposed to polystyrene beads. The LM cells endocytized the beads in direct proportion to the bead/cell ratio and the bead surface area. However, equal volumes of beads irrespective of size or surface area were internalized. The lipid composition of the phagosome membrane differed significantly from the parent primary membrane in having higher contents of phosphatidylcholine, phosphatidylserine, and sterol but lower contents of sphingomyelin and lysophosphatidylcholine. When phagosomes isolated from suspension-cultured LM fibroblasts were exposed to trinitrobenzenesulfonic acid at 4 °C, 55±1.6% of the phagosomal membrane phosphatidylethanolamine was trinitrophenylated. The asymmetric distribution of phosphatidylethanolamine across the phagosomal membrane was not affected by the bead/cell ratio, bead diameter, or exposure time of LM fibroblasts to the beads. When cells were reacted with trinitrobenzenesulfonic acid at 4 °C prior to phagocytosis, the amount of trinitrophenylphosphatidylethanolamine was greater in the isolated phagosomes than in the parent primary plasma membrane. Culturing LM fibroblasts in suspension or monolayer had no effect on the asymmetric distribution of phosphatidylethanolamine across primary plasma membrane bilayers. The data are consistent with the observation that LM fibroblasts grown either in suspension or monolayer internalize polystyrene beads at selective sites in the surface membrane.

Key words phagosomes · endocytosis · plasma membrane · lipid asymmetry · LM fibroblasts · selective sites

Introduction

Almost all eucaryotic cells endocytose. Both normal cells such as macrophages, granulocytes, and neoplastic cells such as Schmidt-Ruffin sarcoma cells, human granulocytic leukemia cells, Burkitt lymphoma cells, L cells, LM fibroblasts, murine neuroblastoma cells, and Ehrlich ascites carcinoma cells demonstrate high phagocytic activity (Roberts & Quastel, 1963; Trouet, Capeneere & deDuve, 1972; Silverstein, Steinman & Cohn, 1977; Sandra & Pagano, 1978; Charalampous, 1979; Evans, Ward & Fink, 1979; Nagpal & Brown, 1980). Endocytosis is involved in transport of maternal proteins to the fetus, in neurotransmission, low density lipoprotein internalization and cholesterol metabolism, removal of damaged cells or foreign material, plasma membrane synthesis by recycling of components from phagolysosomes, secretion, and internalization of parasites such as the protozoan, *Plasmodium sp.* (Silverstein et al., 1977). However, the mechanism of endocytosis is still not understood.

Some investigators believe that endocytosis involves pre-existing nonspecialized areas of the plasma membrane (Evans et al., 1979) and that the surface membrane and phagosomal membrane are essentially identical (Wisnieski & Iwata, 1977; Sandra & Pagano, 1978). In contrast, others have shown that certain lectin receptors are selectively removed from the cell surface while transport proteins are spared (Tsan & Berlin, 1971; Oliver, Ukena & Berlin, 1974). Compartmentalization of cyclic AMP during phagocytosis also occurs at selective sites (Pryzwansky, Steiner, Spitznagel & Kapoor, 1981). Not only may protein-associated functions be selectively associated with phagocytosis, but lipid dynamics as well. Increased synthesis of phosphatidylcholine and phosphatidylinositol occurs upon phagocytosis by polymorphonuclear leucocytes (Sastry & Hokin, 1966; Elsbach, Patriarca, Pettis, Stossel, Mason & Vaughan, 1972). The newly formed phosphatidylcholine was almost all associated with phagosomes. Others have shown selective loss of cholesterol from the surface membrane (Seed & Kreier, 1972; Beach, Sherman & Holz, 1977). The resultant altered cholesterol/phospholipid ratio in the surface membrane of the parent cell also altered the microviscosity of the membrane (Berlin & Fera, 1977; Howard & Sawyer, 1980). These data taken from many endocytic systems indicate that phagosomal membranes may represent a specialized area of surface membrane or that remodeling of the cell's plasma membrane prior to or during endocytosis must take place; but how membrane lipids are involved is not known. This fundamental question is

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of considerable importance since it is not known if altered morphology (Schroeder, Perlmutter, Glaser & Vagelos, 1976; Charalampous, 1979), cholesterol content (Heininger & Marshall, 1979), phospholipid composition (Schroeder, 1981*a*) or degree of fatty acid unsaturation (Roberts & Quastel, 1963; Mahoney, Hamill, Scott & Cohn, 1977; Schroit & Gallily, 1979) affect endocytic rates by diminishing or changing specialized membrane areas at which endocytosis occurs or if some other mechanism such as fusion with lysosomes is involved.

Lastly, endocytosed particles have also been used to represent inside-out plasma membranes, to measure the asymmetric distribution of aminophospholipids across the cell surface membrane, and to determine the physical properties of the two halves of the surface membrane bilayer (Wisnieski & Iwata, 1977; Sandra & Pagano, 1978; Kramer & Branton, 1979; Op den Kamp, 1979; Schroeder, 1980*b*; 1981*b*; Hale & Schroeder, 1982). Thus, it is important to determine if the phagosome actually represents a random or a specialized membrane area of the surface bilayer. In the present investigation, we have determined the lipid composition and asymmetric distribution of phosphatidylethanolamine in both the primary plasma membrane of LM cells and the endocytosed membrane surrounding latex beads. In addition, we have investigated how factors such as culture conditions, latex bead size, bead/cell ratio, and duration of endocytosis affect these parameters. The results of these investigations should provide important new information regarding the origin of the phagosomal membrane.

Materials and Methods

Materials

[$^{32}P_i$] was obtained from New England Nuclear Inc., Boston, Mass. Trinitrobenzenesulfonic acid was purchased from Sigma Chemical Co., St. Louis, Mo. Bovine serum albumin (Fraction V, Pentex, Miles Research Labs., Elkhart, Ind.) was delipidized by the procedure of Wilcox, Dunn and Heimberg (1975). Latex beads were purchased from Dow Diagnostics, Dow Chemical Co., Indianapolis, Ind. Both polystyrene beads (diameter = 0.109, 0.497, 0.760, and 0.945 μm; density = 1.050 g/ml) and polyvinyltoluene beads (diameter = 2.02 μm; density 1.027 g/ml) were used.

Cell Culture

LM cells, a choline-requiring strain of mouse fibroblasts, were obtained from the American Type Culture Collection (CCL 1.2). The cells were grown either in suspension culture or in monolayer culture on 75 cm^2 polystyrene tissue culture flasks (Corning Glass Works, Corning, N.Y.) in a serum-free, chemically defined medium (Higuchi, 1970) as described by Schroeder et al. (1976).

Cells in logarithmic growth were centrifuged at $225 \times g$ for 10 min and the supernatant was decanted (all centrifuged forces refer to r_{av}). The cell pellet was resuspended at 1×10^6 cells/ml in media containing 2 μCi [^{32}P]phosphate/ml (New England Nuclear Inc., Boston, Mass.) and maintained in logarithmic growth phase by the daily addition of the same [^{32}P]-containing medium for 6 days. Thus, the added medium and cell culture medium always had the same specific activity of [^{32}P].

Membrane Isolation

Plasma membranes, microsomes, and mitochondria were isolated from LM cells grown in suspension as described by Schroeder et al. (1976). Plasma membranes were also isolated from LM cells grown in monolayer except that the monolayer cultures were first scraped from the dish with a rubber policeman.

Enzymatic Determinations

The following enzymatic activities were determined in crude homogenates, isolated plasma membranes, and purified phagosomes: Ouabain-sensitive (Na^+, K^+)-ATPase, 5′-nucleotidase, and liberated phosphate were assayed as previously described (Schroeder et al., 1976). TPNH-dependent and succinate-dependent cytochrome *c* reductase were assayed as described by Sottocasa, Kuylenstierna, Ernster and Bergstrand, 1967). Acid phosphatase was assayed using a commercially available kit (Sigma Chemical Co., St. Louis, Mo.).

Phagocytosis and Phagosome Isolation

Phagosomes were isolated by the procedure of Sandra and Pagano (1978) as described by Schroeder (1981*a*). Monolayer and suspension-grown cells were first washed two times with 20 volumes of phosphate-buffered saline [Ca^{++}- and Mg^{++}-free (Schroeder et al., 1976)]. The suspension cells were pelleted as described above while the monolayer cultures were decanted. Unless otherwise specified 5×10^8 cells (monolayer or suspension) were exposed to latex beads (2000 beads/cell) in 30 ml of fresh medium and all other procedures were as described earlier (Schroeder, 1981*a*). After five days' exposure of LM cells to ^{32}P, the phospholipids were uniformly labeled to the same specific activity. Thus, both phagosomal lipid phosphate determination or [^{32}P]phospholipid measurement provided the same information regarding the relative amount of membrane internalized.

Trinitrobenzenesulfonate Labeling

The asymmetric distribution of aminophospholipid across LM fibroblast surface membranes may be determined by using the chemical labeling reagent, trinitrobenzenesulfonic acid, which covalently trinitrophenylates amino groups on phosphatidylethanolamine, phosphatidylserine, or proteins (Fontaine & Schroeder, 1979; Fontaine, Harris & Schroeder, 1979, 1980; Schroeder, 1980*a*; Schroeder, Fontaine, Feller & Weston, 1980). Penetration of the plasma membrane by the reagent was monitored by determination of trinitrophenylation of lipids in intracellular organelles such as microsomes or mitochrondria (Fontaine & Schroeder, 1979). Washed LM cells either cultured in suspension or monolayer were treated with trinitrobenzenesulfonic acid under penetrating conditions (37 °C for 80 min) or nonpenetrating conditions (4 °C for 80 min). The reaction was terminated with TRIS buffer (Fontaine & Schroeder, 1979). The cells were then either exposed to latex beads or plasma membranes were isolated.

Phagosomes were reacted by resuspending the phagosomal pellet obtained in the previous section in 8 ml of 4 mM trinitrobenzenesulfonate labeling reagent (Fontaine & Schroeder, 1979); shaking on a gyrotary shaker for 80 min at 4° or 37 °C; terminating

the reaction with 40 ml 0.15 M TRIS·HCl, pH 7.0; and centrifuging at 33,000 × *g* for 20 min at 4 °C. The washing procedure was repeated one more time with TRIS and one time with phosphate-buffered saline).

Lipid Determinations

All organic solvents were glass-distilled and all glassware was sulfuric acid/dichromate-washed before use. Whole cells, membrane fractions, or phagosomes were suspended in 1.0 ml of the phosphate-buffered saline solution mentioned above. A 0.2-ml aliquot was removed for protein determination (Lowry, Rosebrough, Farr and Randall, 1951). The remaining 0.8-ml aliquot was extracted by the method of Bligh and Dyer (1959) as described by Ames (1966). Before and after silicic acid chromatography, total lipid and total lipid phosphate were determined by the method of Marzo, Chirardi, Sardini and Meroni (1971), and Ames (1966), respectively. Neutral and phospholipids were separated by silicic acid chromatography (Schroeder et al., 1976). The phospholipid composition of whole cells and membrane fractions was determined by exposing the cells to [^{32}P]phosphate (New England Nuclear Crop., carrier free) as described by Schroeder et al. (1976). Fatty acid methyl esters were prepared from phospholipids, separated by silicic acid chromatography as described above, by transmethylation with BF_3 in methanol. The samples were dissolved in hexane and analyzed by gas-liquid chromatography as described previously (Schroeder et al., 1976). Desmosterol content was determined by the method of Sokoloff and Rothblat (1972). Desmosterol (24-dehydrocholesterol), cholesterol (5-cholesten-3β-ol), and coprostanol (5β-cholesten-3β-ol) were used as standards for comparison of retention times by gas-liquid chromatography (Schroeder et al., 1976).

Results

Purification of Phagosomes

LM fibroblasts grown in suspension were exposed to 0.76 μ latex beads for 15 min at 37 °C, and phagosomes were isolated. The phagosomal membrane fraction had the following enzyme specific activities: (Na^+, K^+-ATPase, 59.8 ± 7.4 nmol/min/mg protein and 5′-nucleotidase, 33.8 ± 3.1 nmol/min/mg protein. These values represented a 7.1- and 7.0-fold enrichment, respectively, of these enzyme activities over the cell homogenate. Plasma membranes isolated from the LM cells as described in Materials and Methods had specific activities of these enzymes of 63 and 29 nmol/min/mg protein representing 7.5- and 6.1-fold purification over the cell homogenate. These data indicate a very similar enrichment of plasma membrane enzyme markers in the phagosomes as in the isolated plasma membranes. Acid phosphatase activity was detected in cell homogenate but not in isolated plasma membranes. In phagosomes, the acid phosphatase activity was not detectable during the first 15 min of endocytosis. Longer incubation times (e.g. 60 min) resulted in the appearance of acid phosphatase in the phagosomes, suggesting intracellular fusion with lysosomes. The activities of TPNH-dependent and succinate-dependent cytochrome *c* reductase, microsomal and mitochondrial enzyme markers, were reduced four- and 20-fold, respectively, in the plasma membranes and 4.2- and 18-fold, respectively, in the phagosomes. In addition, it is important to demonstrate that the phagosomal membrane lipids and lipid compositions do not simply reflect the nonspecific adherence of membrane fragments to noninternalized latex beads. It has been shown that at 4 °C phagocytosis is inhibited in several cellular systems (Rabinovitch, 1967; Hoff, Huang, Wisnieski & Fox, 1976). When LM fibroblasts grown in suspension were exposed to 0.76 or 0.49 μ beads for 30 min at 4 °C very little radioactivity was associated with the 10–27% interface on the sucrose gradient at which the phagosomes are found. Less than 5% as much [^{32}P]phospholipid was found as compared to control cells which endocytized the latex beads at 37 °C. Similar data were also found when the LM cells were first homogenized, then exposed to latex beads either at 4 or 37 °C and centrifuged. These results indicate that the latex beads were actively endocytized, that they were purified to a similar extent as plasma membranes, and that the plasma membranes did not nonspecifically adhere to the latex beads during the isolation procedure.

Parameters Affecting Uptake of Latex Beads by LM Fibroblast

In the present investigation endocytic activity was measured by determining the quantity of membrane lipid internalized by the beads. The maximal internalization of polystyrene beads by LM cells occurred very rapidly, reaching a plateau by 10–15 min of exposure of cells to beads over a 10-fold range of bead/cell ratios (Fig. 1). Thus, bead uptake appears linear only for a very short period and other processes such as recycling of internalized membrane back to the plasma membrane may be occurring. Uptake of polystyrene beads by the LM cells was 130 ± 10, 280 ± 28 and 1050 ± 62 cpm [^{32}P]phospholipid/10^6 cells at 200, 800 and 2000 beads/cell. Thus, polystyrene bead phagocytosis was essentially linearly dependent on bead-to-cell ratio.

A number of factors may affect the lipid composition of phagosomal membranes: (1) It is possible that at 5 min a different area of membrane may be internalized by bead uptake than that internalized at 60 min. Alternately, phagosomes may fuse with lysosomes at later time points, thereby changing the phagosomal lipid composition. However, the phospholipid composition of the phagosomes did not change significantly as a function of time (Table 1). (2) At low bead/cell ratios different membrane areas may be in-

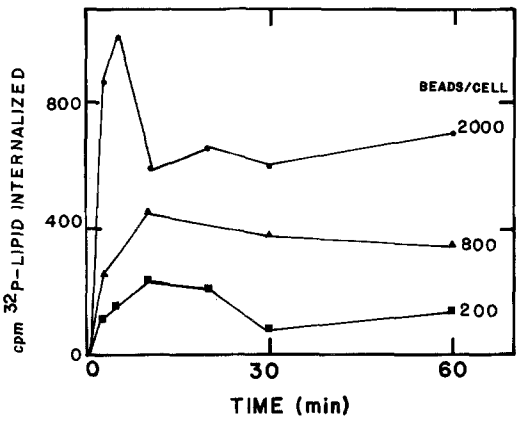

Fig. 1. Effect of polystyrene bead/cell ratio on internalization of LM fibroblasts surface membrane. LM fibroblasts grown in suspension culture for 5 days with [^{32}P] were treated with polystyrene beads (0.790 μ diameter) and internalized membranes were measured by determination of the quantity of [^{32}P]phospholipid associated with the phagosomes. The cells were incubated with latex beads at cell-to-bead ratio of 200, 800 and 2,000 as described in Materials and Methods

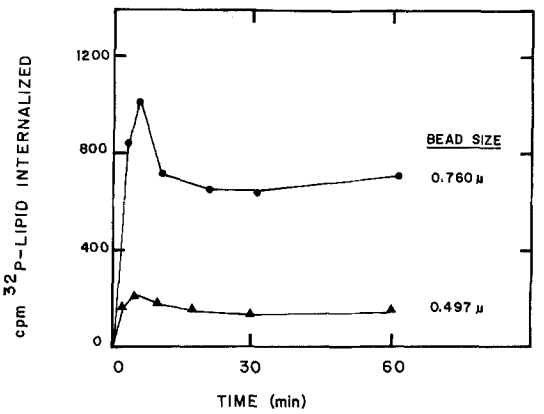

Fig. 2. Effect of bead size on internalization of LM fibroblast surface membrane. All procedures were performed as described in the legend of Fig. 1 except that 0.76 and 0.497 μ diameter beads were used

ternalized as compared to high bead/cell ratios. However, the phospholipid composition of the phagosomal membrane and reactivity of phagosomal phosphatidylethanolamine with trinitrobenzenesulfonic acid were very similar over a 10-fold bead/cell ratio range (Table 2). (3) The effect of bead size on uptake of polystyrene beads is illustrated in Fig. 2. Exposure of LM cells at 2,000 beads per cell to 0.760 and 0.497 μ diameter beads resulted in enhanced internalization of phagosomal membrane [^{32}P]phospholipid. Similarly the [^{32}P]phospholipid internalized/10^6 cells was 4 times higher for 0.760 μ diameter beads (Table 3). If the internalized membranes are compared on the basis of bead area, the 0.760 μ diameter beads internalized twofold more membrane. On the basis of bead volume, however, equal amounts of membrane were internalized. These results were confirmed by measurement of phagosomal membrane phospholipid and by determination of phagosomal membrane protein. Thus, LM fibroblasts internalized

Table 1. Phospholipid composition of phagosome membranes after varying time of phagocytosis[a]

Phospholipid species	% Composition			
	5 min	15 min	30 min	60 min
Phosphatidylcholine	52.0	50.2	50.7	51.9
Phosphatidylethanolamine	30.2	30.2	27.8	26.1
Phosphatidylinositol + Phosphatidylserine	5.4	7.3	7.8	9.9
Sphingomyelin + Lysophosphatidylcholine	5.2	5.2	4.1	4.8
Phosphatidylglycerol	2.1	3.3	1.2	1.5
Other	5.1	3.7	8.4	5.8
Phosphatidylcholine/ Phosphatidylethanolamine	1.72	1.66	1.82	1.99
Anionic/Zwitterionic	0.75	0.81	0.82	0.76
% Phosphatidylethanolamine labeled with TNBS	53	54	52	55

[a] LM cells were exposed to 0.497 μ beads (2000 beads/cell) at 37 °C; at varying time points phagosomes were isolated and treated with trinitrobenzenesulfonic acid (Materials and Methods).

Table 2. Effect of varying the latex bead-to-cell ratio on phospholipid composition of phagosome membranes[a]

Phospholipid species	Bead/Cell ratio			
	200	500	1000	2000
	(% composition)			
Phosphatidylcholine	51.9	53.2	50.3	47.0
Phosphatidylethanolamine	23.5	21.9	24.2	26.5
Phosphatidylinositol + Phosphatidylserine	9.0	8.1	9.4	8.0
Sphingomyelin + Lysophosphatidylcholine	5.3	6.3	6.9	6.6
Phosphatidylglycerol	5.9	4.6	4.5	6.2
Other	4.6	5.9	4.7	5.7
Phosphatidylcholine/ Phosphatidylethanolamine	2.4	2.43	2.08	1.77
Anionic/Zwitterionic	0.75	0.68	0.75	0.87
% Phosphatidylethanolamine labeled with TNBS	55	53	50	51

[a] LM cells were exposed to latex beads (0.497 μ) at varying bead/cell ratio for 30 min at 37 °C. Phagosomes were then isolated, treated with trinitrobenzenesulfonic acid, and lipid composition was determined as described in Materials and Methods.

Table 3. Effects of latex bead size on phagocytosis by LM fibroblasts cultured in suspension[a]

^{32}P Phospholipid internalized	Bead size	
	0.497 μ	0.760 μ
CPM/10^6 cells	122±15	496±50
CPM/10^6 cells/bead area	122±15	212±21
CPM/10^6 cells/bead volume	122±15	138±14

[a] Bead uptake is expressed on the basis of surface area or bead volume compared to the area or volume of 0.497 μ beads. The ratio of surface area of 0.760 μ beads/surface area of 0.497 μ beads was 2.34. The ratio of volume of 0.760 μ beads/volume of 0.497 μ beads was 3.58. Values represent the mean ± SEM ($n=3$).

equal volumes (or mass) of polystyrene beads independent of particle size. No effect of latex bead size on phospholipid composition of the phagosomal membrane was noted (Table 4). (4) The asymmetric distribution of phosphatidylethanolamine across the phagosomal membrane may be dependent on any of the above factors. However, as shown in Tables 1, 2 and 4, neither duration of exposure to beads, the bead/cell ratio, nor bead size affected the asymmetric distribution of phosphatidylethanolamine in the phagosomal membrane.

Table 4. Effect of latex bead size on phospholipid composition of phagosome membranes[a]

Phospholipid species	Bead size (μ)				
	0.109	0.497	0.760	0.945	2.02
	(% composition)				
Phosphatidylcholine	48.3	45.8	51.2	51.6	49.0
Phosphatidylethanolamine	24.6	28.2	26.8	29.0	28.9
Phosphatidylinositol + Phosphatidylserine	8.0	11.0	8.0	6.2	7.7
Sphingomyelin + Lysophosphatidylcholine	6.3	4.9	4.5	4.4	6.0
Phosphatidylglycerol	7.6	4.7	4.1	3.0	6.7
Other	5.2	5.4	5.4	5.8	1.7
Phosphatidylcholine/ Phosphatidylethanolamine	1.96	1.62	1.91	1.78	1.70
Anionic/Zwitterionic	0.83	0.97	0.80	0.79	0.82
% Phosphatidylethanolamine labeled with TNBS	53	53	49	54	52

[a] LM cells were exposed to various sizes of beads (2,000 beads/cell) for 30 min at 37 °C. Phagosomes were then isolated, treated with TNBS, and lipid composition was determined as described in Materials and Methods.

Comparison of Plasma Membrane and Phagosome Phospholipid Composition

Lipids in the phagosomal membranes are derived in large part from the cell surface plasma membrane. Differences in lipid composition of the plasma membranes could give rise to differences in phagosomal membrane lipid composition. The phospholipid composition of plasma membranes from LM fibroblast grown in suspension was significantly different from those grown in monolayer (Table 5). Cells grown in suspension contained more phosphatidylcholine and phosphatidylserine but less phosphatidylglycerol in their surface plasma membranes than did membranes from monolayer cells. The content of phosphatidylcholine was higher while the amount of phosphatidylethanolamine was lower in phagosomal membranes

Table 5. Phospholipid composition of plasma membranes and phagosomes from LM cells cultured in suspension or monolayer[a]

Phospholipid species	Plasma membranes		Phagosomes	
	Suspension	Monolayer	Suspension	Monolayer
	(% composition)			
Phosphatidylcholine	36.6±0.7*	32.2±1.4*	50.6±1.3*	40.9±2.3*
Phosphatidylethanolamine	32.4±1.4	29.7±1.8	24.0±0.9*	29.5±1.7*
Phosphatidylinositol	5.1±1.4	7.8±0.8	4.5±0.6	8.0±1.9
Phosphatidylserine	5.4±1.5*	1.8±0.2*		
Sphingomyelin + Lysophosphatidylcholine	8.4±1.0*	8.1±0.8*	6.3±0.3	7.7±2.6
Phosphatidylglycerol	6.9±1.6*	12.5±1.0*	5.3±0.4	6.1±0.6
Other	5.4±0.8	8.2±1.0	5.4±0.4	4.9±0.5

[a] Phospholipid composition was determined as described in Materials and Methods. Values represent the mean ± SEM, $n=3$. An asterisk signifies $p<0.05$ between monolayer and suspension.

Table 6. Lipid composition of plasma membranes and phagosomes from LM cells cultured in suspension or monolayer[a]

Cell culture	Membrane fraction	Phosphatidylcholine / Phosphatidylethanolamine	Anionic / Zwitterionic	Desmosterol/Phospholipid (mole/mole)
Suspension	Plasma membrane	1.13* (3)	1.00* (3)	0.65±0.10*
	Phagosome	2.11* (6)	0.76* (6)	1.03±0.09*
Monolayer	Plasma membrane	1.08* (8)	1.07 (8)	0.60±0.12*
	Phagosome	1.39* (6)	1.06 (6)	1.48±0.13*

[a] The ratio of anionic to zwitterionic lipids is defined as [phosphatidylcholine + sphingomyelin + lysophosphatidylcholine]/(phosphatidylethanolamine + phosphatidylserine + phosphatidylglycerol + phosphatidic acid + phosphatidylinositol). An asterisk signifies $p < 0.01$ between plasma membrane and phagosome. Values represent the mean ± SEM. Figures in parentheses indicate n.

Table 7. Phosphatidylethanolamine asymmetry in LM fibroblast membranes and phagosome derivatives[a]

Cell culture	Trinitrobenzenesulfonate labeling conditions	Membrane fraction	% Trinitrophenylated phosphatidylethanolamine
Suspension	whole cell	plasma membrane	4.3±0.3 +(3)
	whole cell	phagosome	8.1±0.9* +(3)
	phagosome	phagosome	55.0±1.6* +(16)
Monolayer	whole cell	plasma membrane	4.6±1.0 +(4)
	whole cell	phagosome	13.3±1.6* +(3)
	phagosome	phagosome	63.0±1.3* +(3)

[a] Whole cells or phagosomes were reacted with trinitrobenzenesulfonic acid as described in Materials and Methods. Values represent the mean ± SEM (n). An asterisk signifies $p < 0.05$ between suspension and monolayer, while a double asterisk signifies $p < 0.005$. A plus signifies $p < 0.025$ between plasma membrane and phagosome.

from LM cells grown in suspension than in monolayer (Table 5). Primary plasma membranes of LM cells grown in suspension also had a higher phosphatidylcholine content. Phagosomal membrane phospholipid composition was significantly different from the corresponding parent membrane from cells grown both in suspension and in monolayer, respectively. In both cases phagosomal membranes had more phosphatidylcholine but less sphingomyelin + lysophosphatidylcholine than did the primary plasma membrane. The ratio of phosphatidylcholine/phosphatidylethanolamine was greater in the phagosome than in the primary plasma membranes (Table 6). In addition, the ratio of anionic/zwitterionic lipids was lower in phagosomal membranes than plasma membranes from LM cells grown in suspension but not in monolayer. Phagosomes from both suspension- and monolayer-grown cells were enriched with phosphatidylcholine. This enrichment was greater in phagosomes from suspension-grown LM cells than monolayer-grown LM cells. LM cells cannot synthesize cholesterol but synthesize desmosterol instead (Lengle & Geyer, 1972; Schroeder et al., 1976). The ratio of desmosterol to phospholipid in the phagosomal membranes was higher than in the primary plasma membrane.

Asymmetric Distribution of Phosphatidylethanolamine Across Plasma Membranes and Phagosomes

Phosphatidylethanolamine is asymmetrically distributed across the surface membrane of LM cells grown in suspension. Under nonpenetrating conditions approximately 4% of phosphatidylethanolamine was trinitrophenylated by trinitrobenzenesulfonic acid, while under penetrating conditions greater than 80% was trinitrophenylated (Fontaine & Schroeder, 1979). Under nonpenetrating conditions, 4.3±0.3% and 4.6±1.0% of the phosphatidylethanolamine was trinitrophenylated in the plasma membranes from suspension cells and monolayer cells, respectively (Table 7). If the LM fibroblasts were first exposed to trinitrobenzenesulfonic acid under nonpenetrating conditions, followed by exposure to polystyrene beads, then 8.1±0.9% and 13.3±1.6% of the phosphatidylethanolamine was trinitrophenylated in the phagosomal membrane from cells grown in suspension and monolayer, respectively. This enrichment of trinitrophenylphosphatidylethanolamine was greater in the phagosomes as compared to the plasma membrane for the monolayer cells (2.9-fold) than suspension-grown cells (1.9-fold). Lastly, the percent of phosphatidylethanolamine trinitrophenylated when

Table 8. Fatty acid composition of phagosome derivatives from LM fibroblasts grown in suspension or monolayer[a]

Fatty acid chain length	Phosphatidylcholine	Label whole cells with trinitrobenzenesulfonic acid		Label phagosomes with trinitrobenzenesulfonic acid	
		Phosphatidylethanolamine	Trinitrophenylphosphatidylethanolamine	Phosphatidylethanolamine	Trinitrophenylphosphatidylethanolamine
		Suspension			
16:0	17.7±3.0	11.0±2.7	18.3±2.4	13.4±1.4	19.4±1.5
16:1	8.6±2.5	6.3±1.5	6.8±2.9	8.3±2.1*	9.2±2.0
18:0	19.9±2.6	20.9±1.8*	26.3±3.2*	19.9±2.4	20.5±2.0*
18:1	33.9±3.5*	34.4±4.0*	28.1±2.8*	41.3±3.8* +	23.2±4.1 +
U/S	1.11*	1.19* +	0.90 +	1.39* +	0.86 +
		Monolayer			
16:0	21.5±3.6	20.2±0.8*	11.4±3.0	14.2±1.9	19.1±3.2
16:1	8.7±2.1	4.3±1.4	8.1±2.0	3.1±0.3*	9.8±3.6
18:0	24.2±1.2	34.7±2.9* +	8.4±3.2* +	24.8±4.0 +	5.1±2.1* +
18:1	17.3±3.3*	16.8±3.7*	12.8±3.8*	15.5±3.3*	16.9±3.5
U/S	0.67*	0.50* +	1.02 +	0.66* +	1.05 +

[a] Phospholipid species were isolated and fatty acid composition was determined as described in Materials and Methods. Cells were either first reacted with trinitrobenzenesulfonic acid at 4°, exposed to latex beads, and phagosomes were isolated or cells were first exposed to latex beads, phagosomes were isolated, and phagosomes were reacted with trinitrobenzenesulfonic acid at 4° as described in Materials and Methods. Values refer to the mean±SEM ($n=3$). An asterisk signifies $p<0.01$ ($n=3$) between monolayer and suspension. A dagger signifies $p<0.025$ ($n=3$) between phosphatidylethanolamine and trinitrophenylphosphatidylethanolamine. L/S is the ratio of fatty acid longer than or equal to 18 carbons to fatty acids shorter than 18 carbons. U/S is the ratio of unsaturated to saturated fatty acids.

isolated phagosomes were treated with trinitrobenzenesulfonic acid was 55.0±1.6% and 63.0±1.3% for phagosomes from suspension- and monolayer-cultured LM fibroblasts, respectively.

Fatty Acid Composition of Phagosomal Phospholipids from LM Fibroblasts Grown in Suspension or Monolayer

The acyl composition of phosphatidylcholine and phosphatidylethanolamine from phagosomes isolated from suspension-grown cells was more unsaturated than from phagosomes isolated from monolayer-grown cells (Table 8). In addition, the degree of unsaturation of acyl chains in trinitrophenylphosphatidylethanolamine also differed from that of phosphatidylethanolamine. In phagosomes from suspension-grown cells, the acyl chains of trinitrophenylphosphatidylethanolamine always had a lower U/S ratio than did the acyl chains from the phosphatidylethanolamine. The opposite relationship was determined for acyl chain unsaturation of trinitrophenylphosphatidylethanolamine and phosphatidylethanolamine in phagosomes from monolayer-grown cells.

Discussion

A number of observations were made in this investigation: (1) polystyrene bead uptake by LM fibroblasts was dependent on the bead-to-cell ratio and the time of exposure of the cells to the beads. A constant volume of beads was internalized by the LM fibroblasts. (2) The amount of [^{32}P]phospholipid internalized by the isolated phagosomes was greater with increasing surface area of the ingested latex beads. (3) The phospholipid composition of the phagosomes and the degree of exposure of phosphatidylethanolamine to trinitrobenzenesulfonic acid were independent of exposure time of LM cells to the latex beads, bead/cell ratio, and bead size. These results are consistent with the following concepts: (a) little alteration of the phagosomal membrane phospholipid composition occurred during the first 15 min subsequent to exposure of cells to latex beads; (b) if secondary sites of phagocytosis were generated as a function of time or bead/cell ratio then their phospholipid composition was similar to that of the primary phagocytic sites; and (*c*) these results are consistent with the concept of recycling rather than degradation of phagosomal phospholipids. All three possibilities are in agreement with earlier findings from our laboratory (Schroeder,

1981*a*) and with the results and suggestions of other investigators using neuroblastoma cells or polymorphonuclear phagocytes (Silverstein et al., 1977; Charalampous, 1979).

At least three possible hypotheses may account for the differences between phagosomal and primary plasma membrane lipid compositions determined herein. First, these differences may be due to fusion of lysosomal membranes with phagosomes. However, a number of findings are inconsistent with this possibility: (1) the phagosomal membranes were enriched to the same degree in (Na^+, K^+)-ATPase and 5′-nucleotidase activity as the primary plasma membranes. Neither of these enzymes is enriched in lysosomes (Evans, 1970; Wolff & Pertoft, 1972). If extensive fusion had occurred, then the specific activities of these enzymes in the phagosomes would have been much lower. (2) Intracellular membranes usually have molar sterol/phospholipid ratios lower than 0.3 while plasma membranes have ratios of 0.6 to 1.0. In the present work the phagosomal membranes had sterol/phospholipid ratios of 1.0 to 1.5. Again, if extensive fusion with lysosomes had occurred then the sterol/phospholipid ratio should have been much lower. (3) The phosphatidylcholine/phosphatidylethanolamine molar ratio in lysosomal membranes is 1.8 and up to 2% of the phospholipids are cardiolipin (Strickland & Benson, 1960). In addition, lysosomes contain significant amounts of bis-phosphatidic acid and lyso-bis-phosphatidic acid (Brotherus & Renkonen, 1974). In the present work the phagosomal phosphatidylcholine/phosphatidylethanolamine ratio was 2.1 and 4.1 for phagosomes from suspension- and monolayer-grown cells, respectively. Cardiolipin, bis-phosphatidic acid and lyso-bis-phosphatidic acid were not detectable. (4) The fatty acid composition of phosphatidylethanolamine from lysosomes is enriched in 16:0 (32.8%), 18:0 (34.9%), and 18:1 (10.2%) (Colbeau, Nachbaur & Vignois, 1971). In the present work, the phagosomal fatty acid composition was 16:0 (11.0%), 18:0 (20.9%), and 18:1 (34.4%) for phagosomes from suspension-grown cells. (5) Acid phosphatase activity, a lysosomal enzyme, was not detectable in phagosomes isolated from LM cells 15 min after exposure to latex beads. In summary, the data presented here are inconsistent with the possibility that extensive fusion of phagosomes with lysosomes occurred within the first 15 min of endocytosis, and, if it did occur to a small degree, it did not alter the membrane lipid composition and enzymatic activities significantly.

A second possibility is that phagosomes may be derived from specialized surface membrane areas. The trinitrobenzenesulfonate labeling discrepancy between the plasma membranes and phagosomal membrane may thus reflect the internalization of regions of the plasma membrane in which the normal inside-outside distribution of phosphatidylethanolamine is nonrepresentative of the whole membrane. Several pieces of evidence are consistent with this possibility. The phospholipid composition of the primary plasma membranes from LM cells grown in suspension versus monolayer differed significantly, the former being enriched in phosphatidylcholine and phosphatidylserine but poorer in sphingomyelin, lysophosphatidylcholine, and phosphatidylglycerol. The phospholipids of phagosomes from LM fibroblasts grown in suspension and monolayer were enriched in phosphatidylcholine and sterol. In addition, the molar ratio of phosphatidylcholine/phosphatidylethanolamine in phagosomes from LM fibroblasts grown in suspension was higher than in phagosomes from LM fibroblasts grown in monolayer (2.11 versus 1.39). The ratios of sterol/phospholipid were also higher in phagosomes than in primary plasma membranes. Taken together, these data indicate that the lipids of phagosomal membranes are obtained nonrandomly from specialized phagocytic sites in the surface membrane of the transformed LM fibroblast growing in completely chemically defined media. This possibility is also supported by the work of others (Strickland & Benson, 1960; Tsan & Berlin, 1971; Oliver et al., 1974; Evans et al., 1979; Pryzwansky et al., 1981; Schroeder, 1981*a*). Lastly, recent results of Gupta and Mishra (1981) indicate that phagocytosis of *Plasmodium knowlesi* by erythrocytes leaves a remnant surface plasma membrane with a membrane lipid asymmetry dramatically different from the surface membrane of uninfected cells. Similarly, the sterol/phospholipid ratio of the red blood cell plasma membrane from *Plasmodium berghei* infected cells decreased, as did the microviscosity (Berlin & Fera, 1977; Howard & Sawyer, 1980). If random segments of plasma membrane had been internalized then these properties should not have been significantly altered in the remaining uninternalized plasma membrane.

A third more speculative possibility is that during latex bead internalization extensive lipid architectural rearrangements may occur resulting in a phagosomal membrane with a structure different from an "inside-out" plasma membrane. Either lateral redistribution, altered transbilayer "flip-flop", or synthesis of new lipid may be contributory. This attractive hypothesis is supported by the observation that newly formed phosphatidylcholine is incorporated preferentially into phagosomes during endocytosis (Elsbach et al., 1972). An "inside-out" LM plasma membrane would be expected to have greater than 80% of its phosphatidylethanolamine trinitrophenylated by TNBS under nonpenetrating conditions (Fontaine & Schroeder, 1979). The present data indicate that this was not

the case – only 50–60% of the phagosomal phosphatidylethanolamine was trinitrophenylated. Lastly, the ratio of unsaturated/saturated fatty acids of plasma membrane outer monolayer phosphatidylethanolamine was lower than inner monolayer phosphatidylethanolamine (Fontaine & Schroeder, 1979; Fontaine, Harris & Schroeder, 1979, 1980). If the phagosomal membranes were simply "inside-out" plasma membrane, then the opposite distribution of unsaturated fatty acids should be obtained. This was not true for phagosomes from suspension-cultured or monolayer-grown cells. The latter data did not, however, allow discrimination between possibilities two and three. Thus, both possibilities two and three above are equally tenable. In summary, phagosomal membrane lipids differ from primary plasma membrane lipids in phospholipid composition, acyl chain composition, sterol/phospholipid ratio, and asymmetric distribution of phosphatidylethanolamine. These findings are consistent with a "nonrandom" site of phagocytosis in LM fibroblasts.

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